EVALUATION OF ANTIFUNGAL ACTIVITIES OF EXTRACTS OF SOME MEDICINAL PLANTS ON *NEUROSPORA CRASSA*

MASTER OF PHILOSOPHY

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Evaluation of Antifungal Activities of Extracts of Some Medicinal Plants on *Neurospora crassa*

A THESIS

Submitted in partial fulfillment for the degree of Master of Philosophy to the department of Botany under the faculty of Biological Sciences of the University of Dhaka.

By

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Dedicated

To

My Parents

&

My Aunt

Rawson Ara

CERTIFICATE

This is to certify that this thesis contains the results of research on "Evaluation of antifungal activities of extracts of some medicinal plants on Neurospora crassa" has been carried out by Razia Sultana under our supervision. It is further certified that the work presented here is original and suitable for submission as a M. Phil thesis.

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DECLARATION

I hereby declare that this thesis entitled "**Evaluation of antifungal activities of extracts of some medicinal plants on *Neurospora crassa***" has been composed by me and all the works presented herein are my own. I further declare that this work has not been submitted anywhere for any academic degree.

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Author

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ABSTRACT

Antifungal activity was investigated using the leaf extracts of five different medicinal plants namely, *Ocimum sanctum* Linn., *Coccinia cordifolia* (Linn.) Cogn, *Andrographis paniculata* (Burm. f.) Wall., *Centella asiatica* (Linn.) Urban., *Azadirachta indica* A. Juss. against two fungi *Neurospora crassa* and *Helminthosporium oryzae.* The *in vitro* study revealed the presence of antifungal activity against *Neurospora crassa* in the leaf extracts of all the plants used during the study. The non sterilized leaf extracts was more effective than sterilized leaf extracts. The antifungal effect of these plant extracts enhanced gradually with the application of increased concentration and best antifungal activity was observed in the leaf extracts of *Azadirachta indica* A. Juss. as compared to all the tested plants. It inhibited the radial growth of both *Neurospora crassa* and *Helminthosporium oryzae* at 25% concentration. Antifungal activity was observed in the proteins present in the proteins of third fraction after $(NH_4)_2SO_4$ gradient centrifugation and 200 µg of the total proteins of this fraction was found effective in controlling the fungal growth *in vitro*. Two proteins of the third fraction e.g., 40 and 37 kDa were found to be effective to control the fungal radial growth of both the *Neurospora crassa* and *Helminthosporium oryzae*.

INTRODUCTION

Plants are considered as a source of medicinal agent for thousands of years, which have lead to an increasing interest in the investigation of different extracts obtained from plants as potential source of new antifungal agents. Biologically active compounds present in the medicinal plants have always been a great interest to scientists working in this field. In recent years, this interest to evaluate plants possessing antibacterial and antifungal activity against various common pathogens has been increased. All parts of plants including stem, root, flower, bark, leaves possess antimicrobial and antifungal property (Baris *et al.* 2006).

Plants are the most important source of chemical compounds. Through primary and secondary metabolism plants synthesis essential compounds which have different properties including beneficial and harmful. There is growing evidence that many plant derived compounds when applied on other plants, can protect the plant from photogenes. In the search of environmentally safer, selective and durable natural fungicides, structure identification of components such from various prats of plants is required.

Bangladesh is a great treasure of medicinal plants. More than 500 plants growing in Bangladesh have been reported to posses medicinal properties of some description or other and have been enumerated in the literature as indigenous drugs. Until now only a small part of plant kingdom (estimated at 2,50,000-5,00,000 species around the world) has been investigated phyto-chemically and fraction subjected to biological and pharmacological screening is even lower. After development of multimedia techniques natural resources have been used to be the potential source for safe, biodegradable and more beneficial drugs, remedies, fungicides or pesticides for a sustainable environment on the planet. Fungus, algae, insects or even micro-organisms have also been subjected to yield active compound in this regard. But plants are the most suitable source for such of an interesting propagation in field of fungicides technology while some plants in different parts of the world are considered as toxic and some are used as traditional medicine. A literature search on the medical plant offered some essential opening that this species bears repellent and toxicological properties, and it is then subjected to go through screening and then isolation, purification and structure elucidation of essential constituents to develop natural biodegradable fungi control agent(s). The approaches adopted to obtain and exploit pure plant constituent involves interdisciplinary works in botany, zoology, pharmacology, chemistry and toxicology a described (Hostettman *et al*.1995).

In the time being, most parts of the world, especially in the third world countries the food deficit and quality is one of the biggest problems related to crop protection. The loss of food grain during storage due to various fungal diseases is a serious problem. More than 2000 pest species during the field storage annually destroy approximately one third of world food production, valued at more than $100 million, among which highest losses (43% potential production) occur in developing Asian countries (Ahmed and Grainge, 1986). Annual post harvest losses resulting from fungal damage, microbial deterioration and other factors are estimated to be 10-25% of worldwide production. To overcomes these problems, the fungicides were widely used in the grain storage and crop field and achieved good results, but these agricultural chemicals pollute the grain and decrease their qualitative characters including color, smell and taste. Climate and storage conditions especially in tropics are highly favorable for fungus growth and development. Control of this fungus growth by chemical fungicides has serious drawbacks. The indiscriminate use of chemical fungicides has given rise to many obvious serious problems including genetic resistance by best species, toxic residues, increasing cost of application, environmental pollution, hazard from handling etc (Ahmed *et al.* 1981).

Fungus, bacteria, insects are a problem in stored grain field crops throughout the world because they reduce the quantity and quality of grain. Chemicals largely used as fungicides in crop protection are proved as environmental pollutants and have undesirable effects on animal and human beings in the long run. Therefore, the development of bio-fungicides has been focused as a viable fungal control strategy in recent years. Over 2000 species of plants representing 170 odd families are said to have some antifungal and antipathogenic values. Since Bangladesh has a large sum of plants and an age –old traditional practice to use plants to protect fungal diseases, it is optimistic to have investigation on the promising plants for biologically active compounds for the future ingredients of nature-friendly fungicide(s). Since the use of plant materials for storage purposes is sustainable and biodegradable, they can be continuously propagated year after year, and do not have any negative impact on the environment as long as care is taken to avoid the propagation of plants from foreign ecosystems which might, therefore, become established as weeds. Nevertheless, many plants commonly known as safe may contain noxious compounds, which may render them unsafe for both animals and humans to consume. Now bioactive principles from natural origin are subjected to investigate for fungi control agents as well as for remedies of diseases without residual or side effects. Since plants may contain hundreds or even thousands of metabolites

there is currently a resurgence of interest in the vegetable kingdom as a possible source of new lead compounds for introduction into therapeutical screening program (Hostettmann *et al*. 1995 Prakash and Gupta 2005).

In fact, plant species has a vast repository of chemical substances that protect plants from attacks by various fungi. Some of these chemicals may repel or kill the fungus or deter their reproduction. These properties of the plants are of a great value in protecting stored commodities from fungus.

Natural plant products or botanical fungi control agents that repel or inhibit fungal growth offer considerable potential for crop protection, because repellent activities are highly species dependant and phytochemicals are generally readily biodegradable. So these products extracted on large scale may also be used to replace or supplement the activity of existing synthetic fungicide. Plant derived compounds may offer potential leads for novel agents against systematic fungal diseases of plants and man.

Being situated in the tropics, Bangladesh has a rich biodiversity rich enough and a huge number of her plants are being used in the traditional system of folk medicine, as well as in the control of fungal diseases. In this proposition a primary screening has been done to trace out presence of biologically active components in different parts of indigenous plants and the plants found to contain bioactive potential agents (especially of antifungal activity) given special reference in this dissertation. *Ocimum sanctum* Linn. *Coccinia cordifolia* (Linn.) Cogn., *Andrographis paniculata* (Burm. f.) Wall., *Centella asiatica* (Linn.) Urban., *Azadirachta indica* A. juss. had been taken into consideration as the title experimental plants.

In nature all the plants are under constant threat due to different types of disease caused by fungi, bacteria, virus, nematodes as well as insects. One of the most commonly practiced methodsof controlling these plant pathogens is the use of fungicides. During later half of this century a large number of synthetic inorganic and organic fungicides have been developed to control these plant diseases. However the use of many of such fungicides has now been cautioned due to their carcinogenicity, teratogenicity and other residual toxicities (Bajaj and Ghos 1975). Further, due to the development of new pathogens, many of the synthetic fungicides are gradually becoming ineffective (Wellman 1977). Moreover, the use of these fungicides has also been restricted due to their various side effects such as acute toxicity, long degradation periods, their concentration in the food chain and undesirable extension of their power to kill both useful and harmful organisms (Beye 1978). The excessive use of synthetic

fungicides may pose potential health hazards not only to livestock and wildlife but also to fishes, birds mammals and even to human beings (Rangaswami 1981). Now a day an increasing awareness of the negative side effects of fungicides particularly the inorganic ones of the ecosystem and growing interest in pesticides free agriculture have stimulated the search for naturally occurred fungitoxic compounds which can be used against various fungal plant pathogens.

With the increasing awareness of the problems and expenses of conventional method of disease control, biological control of plants pathogenic fungi has offered an attractive alternative and has now become one of the most exciting and rapidly developing areas in the plant pathology because it has great potential to solve many agricultural and environmental problems.

In recent years, some researches on the fungi toxicity from extracts of various parts of higher plants have indicated the possibility of their exploitation as natural fungitoxicants for controlling plant disease (Thapliya and Nene 1957, Ahmed and Agnihorti 1972, Misra and Dixit 1977, Lokendra and Sharma 1978, Kishore *et al*. 1982, Ashrafuzzaman *et al*. 1990, Bashar and Rai 1991, Anwar *et al*. 1994). Plant extracts are easily degradable having almost no harmful residual effects (Fawcett and Spencer 1970).

Extracts of many higher plants have been reported to exhibit antibacterial, antifungal properties under laboratory trails. Plants metabolites and plant based pesticides appear to be one of the better alternatives as they are known to have minimal environmental impacts.

Plants extracts are cheap, can be easily prepared and used whenever required. In fact some workers have already demonstrated the successful use of the plant. Parts of various higher plants against some important disease of crop plants (Lapis and Dumancus 1978, Ahmed and Sultana 1984, Miah *et al*. 1990).

Thus an attempt was made to evaluate the plant extracts of some selected higher plants such as *Ocimum sanctum* Linn. (Tulshi), *Coccinia cordifolia (*Linn). Cogn (Telakucha), *Andrographis paniculata* (Burm.f.) Wall. (Kalomegh), *Centella asiatica* (Linn.) Urban. (Thankuni), *Azadirachta indica* A. Juss. (Neem) with a view to compare their value as fungitoxicants against *Helminthosporium oryzae* known to cause significantloss in the field and during storage of rice crop. It is hoped that this study will be helpful in suggesting

whether plant extracts of common species of higher plants could be used instead of chemical fungicides against the plant pathogens.

Ocimum sanctum Linn. very commonly known as Tulsi belonging of family Lamiaceae is a time-tested premier medicinal herb It is a plant of Indian origin and apart from its religious value it is used in Ayurvedic medicine since ancient times. It is herb used to treat a variety of illness ranging from diabetes, arthritis, bronchitis, throat infection, skin diseases etc. Its antimicrobial property has been tested against variety of microorganisms (Geeta *et al.* 2001).

Neem (*Azadirachta indica*) tree has attracted worldwide prominence owing to its wide range medicinal properties. Neem leaf and its constituents have been demonstrated to exhibit immunomodulatory, anti-inflammatory, antihyperglycaemic, antiulcer, antimalarial, antifungal, antibacterial, antioxidant, antimutagenic and anticarcinogenic properties. Neem elaborates a vast array of biologically active compounds that are chemically diverse and structurally variable with more than 140 compounds isolated from different parts of the tree. Quercetin and ß- sitosterol, were the first polyphenolic flavonoids purified from neem fresh leaves and were known to have antibacterial and antifungal properties (Hari *et al*. 1998).

Centella asiatica (Linn.) Urban. belongs to the family Apicaceac. *Centella asiatica* (Linn.) Urban. is one of the chief herbs for treating skin problems, to heal wounds, for revitalizing the nerves and brain cells. The use of *Centella* in food and bevarages has increased over the years basically due to its healths benefits such as antioxidant, as antiflammatory, wound healing, memory enhancing property and many others. The potential of *Centella* is an alternative natural antioxidant especially of plant origin and its protection age-related changes in brain antioxidant defense system have notably increased in recent years (Singh *et al.* 2010).

Ethanolic and petroleum extracts of *Centella asiatica* plant shows significantly higher rate of antifungal activity against various fungal strains. Methanolic extract of *Centella asiatica* also showed significant inhibitory effect on spore germination against various fungal strains like *Alternaria, Cercospora, Curvularia, Drechslera* and *Fusarium*. The inhibitory effect on spore germination of the above fungus strains was increased proportionately with the increase in the concentration of methanolic extracts of the leaves. Bobbarala *et al*. examined the antifungal activity of forty nine plants including *Centella* against *Aspergillus niger* fungi using agar well diffusion method. Among the 49 plants studied the methanolic extracts of 43

plants including *Centella* exhibited varying degrees of inhibition activity against the above fungi. Methanol, chloroform and acetone extracts of *Centella asiatica* showed significant inhibitory effect on growth and sporulation of *Colletotrichum gloeosporioides.*

Andrographis paniculata (Burm. f.) Wall. belongs to the family Acanthaceae. *Andrographis paniculata* is one of the most widely used plants in ayurvedic formulations and also in Chines medicine. The Indian pharmacopoeia narrates that *Andrographis paniculata* is apredominant constituent of at least twenty six ayurvedic formulations. *Andrographis paniculata* is one among the prioritized medicinal plants in India and this herb is being used mainly for treating fever, liver disease, diabetes, snake bite.It is also used as antibiotic, antiviral, antimicrobial, antifungal, anti inflammatory, anticancer, anti- HIV, anti-allergic. It is also utilised for common cold, heoatoprotective activity, antimalarial, antidirrheal and intestinal effect, cardiovascular activity, antifertility activity, pain reduction (Chowdhury *et al*. 2012).

Bangladesh has fertile land for cultivation throughout the year. Rice is the most important cereal crop in Asia producing about 96% of the world rice production (IRRI, Rice knowledge Bank 2013). It is the staple food for about 140 million people of Bangladesh. It provides about 75% of the total calories and 55% of the total protein in a typical diet in Bangladesh (BBS 2013).

In Bangladesh 4.11 million hectares of cultivable land produce more than 4.64 million metric tons of rice (BBS 2013). The Average world production of rice is 3.75 metric ton per hectare but the average yield of our country is lamentably poor, only 1.13 metric tons per hectare (FAO 2002, BBS 2013). So the average per hectare production of rice in Bangladesh is extremely low as compared to other rice growing countries of the world.

Low yield of rice is altributed to various factors. Among them vulnerability of crop to pests and diseases is important one. Rice diseases caused by different groups of pathogenic microorganism are virus, bacteria, fungi and nematodes. Thirty six fungal, twenty one viral, six bacterial and six nematode diseases have been recorded in rice (Ou 1985). In Bangladesh thirty one rice diseases have been so far identified of which ten are considered as major (Miah and Shajahan 1987, Anon 1995). Among them Brown leaf spot of rice caused by *Helminthosporium oryzae* rice had been considered as most important because of its wide spread occurrence and significant damage potential.

Brown leaf spot of rice caused by *Helminthosporium oryzae* is one of the major fungal diseases of rice which occurs in almost all the rice growing areas (Singh 2005). The disease is of great importance in several countries and has been reported to cause considerable losses. It occurs, occasionally as an epidemic disease every year in mild or severe form.

On average, the disease causes 5% yield loss across all lowland rice production in South and Southeast Asia. Severely infected field might have as high as 45% yield loss. Heavily infected seeds cause seedling blight and lead to 10−58% seedling mortality. It also affects the quality and the number of grains per panicle, and reduces the kernel weight. Brown spot was considered to be the major factor contributing to the Great Bengal Famine in 1943 (IRRI, Rice knowledge Bank, 2013).

Several chemicals have been reported to control the disease (Bisht and Khulbe 1995). The spraying of the fungicides has been reported to reduce the disease severity effectively (Chhetry 1993). However, the indiscriminate use of chemical fungicides to control the disease is not only hazardous to living beings but also adversely affects the environment (Ansari 1995). These results to find out an alternative approaches which are economically feasible and eco-friendly like botanical pesticides or biological agents to control the disease (Ansari 1995). The present investigation was therefore undertaken to test the antifungal activity of certain plant extracts against *Drechslera oryzae*, the causal organism of brown leaf spot of rice.

Use of plant extracts against plant disease is however, a recent approach to plant disease control. It helps to avoid environmental pollution by chemicals. Successful use of plant extracts in controlling fungal pathogens has been demonstrated by a number of authors (Assadi and Behroozin 1987, Singh 1987, Miah *et al*. 1990, Ashrafuzzaman *et al*. 1992, Hossain 1993).

Biological control is the most accepted, eco-friendly method to control any type of the fungal diseases because this method has not any negative impact on the environment that causes environmental degradation. Plants extract play an important role to check the growth of various fungi. Recently some workers Tripathi *et al*. 1978, Locandula 1993, Solsoloy *et al*. 1993, Kale *et al*. 1995, Garcia and Padilla 1996 all over the world have been studying the botano-chemicals for such purpose. Presently scientists are interested to evaluate the antifungal activity of plant extracts against plant pathogenic fungi. Several workers have

studied the antifungal properties of some medicinal plant extracts against some fungi (Haque and Shamsi 1997). So, the present study was aimed to evaluate the antifungal activity of the leaf extracts of some commonly used medicinal plants namely *Ocimum sanctum* Linn. (Tulshi), *Coccinia cordifolia* (Linn). Cogn (Telakucha), *Andrographis paniculata* (Burm.f.) Wall. (Kalomegh), *Centella asiatica* (Linn.) Urban. (Thankuni), *Azadirachta indica* A. Juss. (Neem). During the study a non-pathogentic laboratory strain named *Neurospora crassa* and plant pathogenic fungi named *Helminthosporium oryzae* were used as target organism.

Aims and objectives of these work:

Evaluation of antifungal activities of some medical plants in order to identify as an effective fungicide and isolation of its active ingredients by-

1. Identification of the best plant extracts by studying the radial growth of *Neurospora crassa* cultured in VM (Vogel 1956) containing aqueous extracts of some selected medical plants.

i. *Ocimum sanctum* Linn.

ii. *Coccinia cordifolia* (Linn.) Cogn.

iii. *Andrographis paniculata* (Burm.f.) Wall.

iv. *Centella asiatica* (Linn.) Urban.

v. *Azadirachta indica* A. Juss.

2. Isolation of the active ingredients responsible for antifungal activity from the particular medicinal plant.
3. Use of the best plant extract to control *Helminthosporium oryzae* a common crop plant pathogen of rice in order to check its efficiency as a fungicide.

MATERIALS

1.1. FUNGAL STRAINS

In the present study, two different fugal strains were used as as target organism for studying antifungal activity of different palnt extracts.

a) *Neurospora crassa* was used as one of the experimental tool since it is non-pathogenic to human and easy to grow in the laboratory condition. Culture Emerson "a" (Ema) (5297), Emerson "A" (EmA) (5296) were used (figure 1). Both the were received by the department of Botany, University of Dhaka from Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Centre, USA. *Neurospora crassa* were kindly supplied by Prof. Dr. Tahsina Rahim from Microbial Genetics laboratory Department of Botany, University of Dhaka.

b) *Helminthosporium oryzae* (figure 2) kindly supplied by Prof. Dr. Shamim Shamsi from plant pathology and mycology laboratory, Department of Botany, University of Dhaka was used as another experimental material.

1.2. PLANT MATERIALS

The plant parts were collected from the Dhaka University Carzon Hall Botany Garden. 50 grams of leaves were measured from each plant to collect extract. The following higher plants were used:

a) Plant name: Tulshi (figure 3)
   Scientific Name: *Ocimum sanctum* Linn.
   Useful Part: Leaf
   Chemical Constituents: *Ocimum sanctum* Linn. leaves yield a bright yellow essential oil containing phenols, aldehyde, eugenol, carvacrol, methyl eugenol, cineol, linalool and caryophyllene. This plant also contains alkaloids, glycosides, flavonoids,

saponins, citric, tartaric and malic acids. It has antifungal activities and is antiseptics (Gani 1994).

b) Plant name: Telakucha (figure 4)
Scientific name: *Coccinia cordifolia* (Linn.) Cogn.
Useful part: Leaf

Chemical Constituents: Aerial parts contain protein fat, vitamin C, sterols, β-sitosterol, phenolic compounds, triterpenoids, bitted glycosidic constituents, alkaloids, cephalandrine A, and B, alcohol, cephalandrol, tritriacontane, heptaacosane (Gani 1994).

c) Plant name: Kalomegh (figure 5)

Scientific name: *Andrographis paniculata* (Burm.f.) Wall.

Useful part: Leaf

Chemical constituents: The plant contains aresinous substance kalmeghin, a bitter crystalline diterpene lactone, andrographolide, diterpen glucoside, neoandrographolide, deoxyandrographolides, epigenin ethers and other flavonoids and phenols. Leaves contain β-sitosterol glycoside, andrographolide and panicolide and polyphenols, caffic and chlorogenic acids and a mixture of dicaffeolquinic acid. Roots contain flavones, andrographin and panicolin, ∝-sitosterol (Gani 1994).

d) Plant name: Thankuni (figure 6)
Scientific name: *Centella asiatica* (Linn.) Urban

Useful part: Leaf

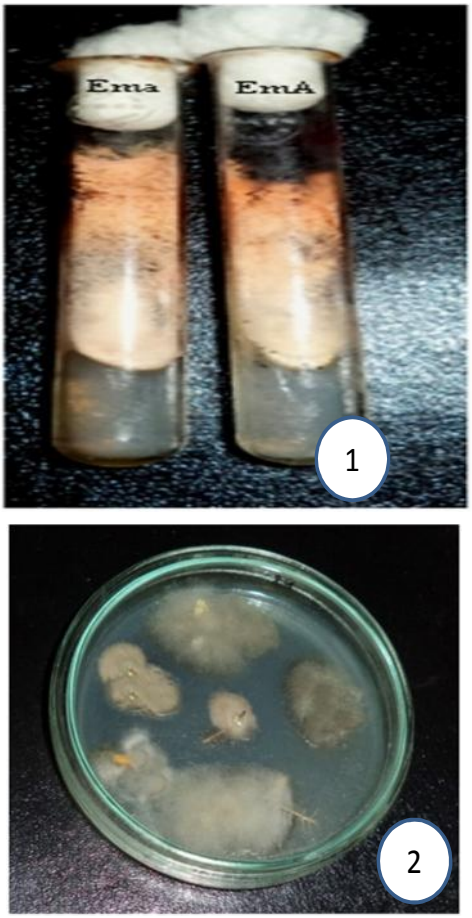
Chemical constituents: The plant principally contains tri teropenes and triterpene glycosides, thankuniside and iso-thankuniside, Asiatic asid and asiaticosside, madecassic acid and madecassoside and ascorbic, thankunic, pectic and brahmic acid. It also contains small amount of resin, tannin, saponins, anthraquinione glycosides, sterols, sitosterol, essential oil, fatty oil, bitter principle, vellarine and an alkaloid hydrocotyline (Gani 1994).

e) Plant name: Neem (figure 7)

Scientific name: *Azadirachta indica* A. Juss.

Useful part: Leaf

Chemical constituents: Various parts of the plant and the neem oil contain bitter principles, saponins and alkaloids. The bitter principles of neem include nimbidin, nimbin, nimbinin, nimbidol and bakayanin. In addition to these, the fruits contain triterpenoids, salanin and azadirachtin, an oil, organic acids and alkaloid, melianon. Leaves contain nimbinene, nimbolide, quercerin and its glycosides, β-sitosterol, n-hexacosanol, nonacosane, ascorbic acid and amino acids. Barks contain tannin, margosin and azadarin. Neem oil contains margosic acid. Flowers contain essestial oil, kaemferol, glucoside, nimbosterin and N- nonacosane. Kernel contains triterpenoids, salanin, azadirachtin, oil and fatty acids. Seeds contain 6 tetranortriterpenes. Flowers contain kaempferol (Gani 1994).



Figs 1-2: figure 1- *Neurospora crassa,* figure 2- *Helminthosporium oryzae*

Figs 3-7: Figure 3: Tulshi plant, Figure 4: Telakucha plant, Figure 5: kalomegh plant, Figure 6: Thankuni plant, Figure 7: Neem plant

1.3. APPARATUS AND GLASS WARES WHICH WERE USED FOR THE PRESENT RESEARCH WORKS ARE AS FOLLOWS-

A. APPARATUS

i) Autoclave machine
ii) Centrifuge machine
iii) Mortal and pestle
iv) Fluorenscence Microscope
v) Incubator
vi) Laminar airflow
vii) Refrigerator
viii) Water bath
ix) Digital balance
x) pH meter
xi) Spectrophotometer
xii) DC voltage power supply mechine
ii) Ice machine
i) Water de-ionizer
ii) Micropipettes and micropipette tips
iii) Eppendrof tubes
iv) Gloves
v) Water distillation plant
vi) Micro-oven
vii) Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad)

B. GLASS WARES

Table 1. List of the glass wares

SL No	Name	Made by	Size	Purpose
1.	Test tubes	Pyrex	0.5″ x 3″	Sub culturing tube
2.	Test tubes	Pyrex	5/8″x 5″	Stock tubes
3.	Petridishes	Pyrex	3″ dia	Media
4.	Flasks (Erlenmeyer)	Pyrex	100ml	Media and water etc
5.	Flasks (Erlenmeyer)	Pyrex	250ml	Media and water etc
6.	Flasks (Erlenmeyer)	Pyrex	500ml	Media and water etc
7.	Flasks (Erlenmeyer)	Pyrex	1000ml	Media and water etc

1.4. CHEMICAL USED FOR THE RESEARCH WORK

A. CHEMICALS FOR VOGEL'S MINIMAL MEDIA

i) Agar

ii) Ammonium Nitrate (NH_4NO_3)

iii) Ammonium Sulfate (NH_4SO_4)

iv) Biotin ($C_{10}H_{16}N_2O_3S$)

v) Boric Acid (H_3PO_4) anhydride

vi) Calcium Chloride ($CaCl_2$. $6H_2O$)

vii) Chloroform ($CHCl_3$)

viii) Citric Acid [$C(OH)(COOH)(CH_2COOH)_2.H_2O$]

ix) Copper Sulfate ($CuSO_4.7H_2O$)

x) Ferrous Sulfate ($FeSO_4.7H_2O$)

xi) Glucose ($C_6H_{12}O_6$)

xii) Manganese Sulfate ($MnSO_4.7H_2O$)

xiii) Magnesium Sulfate ($MgSO_4$. $7H_2O$)

xiv) Hydrochloric Acid (HCl)

xv) Magnesium Chloride (MgC_{l2})

xvi) Methanol (CH_3OH)

xvii) Potassium Nitrate (KNO_3)

xviii) Potassium di-hydrogen phosphate (KH_2PO_4)

xix) Sodium Chloride (NaCl)

xx) Sodium Citrate ($NaH_2C_6H_5O_7.2H_2O$)

xxi) Sodium Molybdate ($Na_2MoO_4.2H_2O$)

xxii) Sucrose ($C_{12}H_2O_{11}$)]

xxiii) Zinc Sulfate ($ZnSO_4.5H_2O$)

xiv) Dextrose

B. CHEMICALS FOR PHOSPHATE BUFFERED SALINE (PBS BUFFER)

i) Sodium Chloride (NaCl)

ii) Potassium Chloride (KCl)

iii) Sodium di-hydrogen phosphate (Na_2HPO_4)

iv) Potassium di-hydrogen phosphate (KH_2PO_4)

C. CHEMICALS FOR POLYACRYLAMIDE GEL ELECTROPHORESIS PREPARATIONS

i) Tris-HCL
ii) Acrylamide
iii) Bisacrylamide
iv) Amonium Persulfate (AP)
v) Tetramethylethylenediamine (TEMED)
vi) Tris-Base
vii) Beta mercapto-ethanol
viii) Glycerol
ix) Bromophenolblue
x) Glycine
xi) Sodium Dodecyl Sulphate (SDS)

D. REAGENTS USED FOR ESTIMATION OF PROTEIN CONTENT

Reagent -1

i) 1% NaOH + 1% anhydrous Na_2CO_3

ii) 1% Cu SO_4. $5H_2O$

iii) 2% Na-K –tertrate

Reagent-2

Folin and ciocalteu's phenol

MEDIA

Wild type strains of *Neurospora crassa* can be grown on a suitable mixture of mineral salt in water plus carbon sources (sucrose, glucose) and the vitamin biotin (Beedle and Tatum 1945, Anonymous 1996).

To prepare VM media different stock solution were needed. Conical flask was used for keeping stock solution. The chemicals were added one after another and the addition of the next one was followed by the complete dissolution of the previous one. All the stock solutions were kept in the refrigerator. All media were sterilized in the autoclaved at 121°C under 15 Ib pressure for 20 minutes.

2.1. Composition of Biotin solution per 100ml

i) Distilled Water 100ml

ii) Biotin 10 mg

The solution was used for the preparation of Vogel's stock solution

2.2. Composition of Trace element solution per 100ml

i) Distilled water	95 ml
ii) Citric Acid [$C(OH)(COOH)(CH_2COOH)H_2O$]	5g
iii) Zinc Sulfate ($ZnSO_4.7H_2O$)	5g
iv) Ferrous Sulphate ($FeSO_4.6H_2O$)	1.0g
v) Copper Sulphate ($CuSO_4.5H_2O$)	0.25g
vi) Manganese Sulfate ($MnSO_4.H_2O$)	0.65g
vii) Boric acid anhydrouas (H_3BO_4)	0.05g
viii) Sodium Molybdate ($NaMoO_4.2H_2O$)	0.05g
xi) Chloroform ($CHCl_3$)	1ml

The solution is needed for the preaption of Vogel's stock solution.

2.3. Composition of Vogel's stock solution per litre

i) Distilled water	770 ml
ii) Sodium Citrate	63.5g
iii) Potassium dihydrogen phosphate (KH_2PO_4)	125mg
iv) Ammonium Nitrate (NH_4NO_3)	50g
iii) Magnesium Sulphate ($MgSO_4.7H_2O$)	5g
iv) Calcium Chloride ($CaCl_2.6H_2O$)	3.75g
v) Trace element solution	2.5ml
vi) Biotin solution	1.5ml
vii) Chloroform ($CHCl_3$)	2ml

Vogel's stock solution was essential for the preparation of Vogel's minimal medium. The stock solution was kept in 1000ml conical flask. Calcium chloride was added first to the distilled water then other chemicals were added one after another.

2.4. Vogel's minimal medium

The medium was used for culturing *Neurospora crassa* in the test tubes and radial growth on theplates. For maintaining cultures 2-4 ml of medium was taken in small (3″) test tubes and 10-15ml of medium was taken in petridishes for cultures growing spores.

2.5. Composition of Vogel's minimal medium (VM) per liter for culturing

i) Vogel's stock solution	40ml
ii) Distilled water	960ml
iii) Agar	15g
iv) Sucrose	20g

The medium was used for sub-culturing in case of plating 2% agar was used, 2% glucose was used in lieu of sucrose.

2.6. Composition of Potato Dextrose Agar PDA medium

i) Potato (Peeled and sliced)	200 gm
ii) Dextrose	20 gm
iii) Agar	15 gm
iv) Distilled water	1000ml
v) pH	5.5

The peeled and sliced potato in 500 ml distilled water was boiled for half an hour. At the same time the agar was melted in 500ml of distilled water. The potato extract was strained into melted agar and the volume was adjusted with the distilled water. Dextrose was added before the medium was autoclaved.

Potato Dextrose Agar (PDA) medium was used for cutting and platting of *Helminthosporium oryzae*.

2.7. Sterilization

All the media, distilled water were sterilized in the autocalved at 121°C under 15 Ib pressure for 20 minutes. The inculation chamber, needle and the working space were sterilized with rectified spirit and the needle was burnt over the flame with a sprit lamp.

METHODS

3.1. SUBCULTURING

To obtain fresh cultures and for preservation, subculturing was important. Vogel's minimal medium was used for subculturing wild types (Em 'a' and Em 'A') of *Neurospora crassa.* Potato Dextrose Agar (PDA) medium was used for subculturing of *Helminthosporium oryzae*. Generally small tubes containing 1-2 ml of sterilized media were used for subculturing.

Subculturing was done in a sterilized laminar airflow. The cabinet, the inoculation needle and the hands were first sterilized with rectified spirit. The inoculation needle was sterilized by buring over the flame of a sprit lamp kept in the chamber until the needle become into red and then cooled in the medium before each time of transferring the inoculum. Inoculum from the old stock was transferred to new medium very carefully to avoid contamination. Then the tubes carrying inoculums were incubated at 25°C for the growth and conidiation.

3.2. STOCK MAINTAINING

The stock of the cultures of the wild types *Neurospora crassa* (Em 'a' and Em 'A') and *Helminthosporium oryzae* were maintained in stock tubes containing 5 ml medium. The wild type of *Neurospora crassa* (Em 'a' and Em 'A') were grown in Vogel's minimal medium and *Helminthosporium oryzae* was grown in Potato Dextrose Agar Media (PDA). After incubation period, when cultures produce sufficient conidia were preserved in a refrigerator at ± 4°C. The cultures were found to produce sufficient conidia within 4 to 5 days.

3.3. STUDY OF EFFECT OF EXTRACTS OF *Ocimum sanctum* Linn.

A. PREPARATION OF LEAF EXTRACTS OF *Ocimum sanctum* Linn. (TULSHI)

Collected leaf samples were rinsed with running tap water followed by washing with distilled water and air dried. 50gm of fresh leaves of *Ocimum sanctum* Linn. (Tulshi) was measured with the help of a balance and crushed to fine powder in mortar pestle using liquid N_2 or keeping in ice. The paste was collected in a falcon tube and centrifuged for 10 minutes at 4°C. Then the supernatant was collected into another falcon tube. This preparation was done twice and one set was sterilized in the autoclave at 121°C under 15 Ib pressure for 20 minutes and other set was kept at 4°C incubator to use as non sterilized from. The overall protocol has been presented in the following flow chart (Fig.8).

50gm leaves of examined plant were washed with sterilized distilled water

↓

Air dried

↓

Weight was noted

↓

Grinded/crushed with Liquid N_2 in mortar pestle

↓

Centrifuged (10 minutes at 4°C)

↓

Supernatant was collected and used asplant extract

Figure 8. Generalized flowchart showing the step by step method of preparing extracts from leaf

B. PREPARATION OF DIFFERENT SOLUTIONS OF *Ocimum sanctum* Linn. LEAVES

Different concentrations (25, 50, 75 and 100%) of leaf extract of *Ocimum sanctum* Linn. solutions with sterilized distilled water were prepared (Table 2).

Table 2. Different concentrations of *Ocimum sanctum* Linn. extract solution.

Name of the solutions	Amount of crude extracts	Amount of sterilized distilled water	Concentration of the crude extract in the solution in %
O_1	1ml	3ml	25%
O_2	2ml	2ml	50%
O_3	3ml	1ml	75%
O_4	4ml	0ml	100%

The prepared solutions were kept separately in sterilized falcon tubes.

C. EVALUATION OF THE EFFECT OF NON-AUTOCLAVED (NORMAL) AND AUTOCLAVED LEAF EXTRACT OF *Ocimum sanctum* Linn. (TULSHI) AGAINST THE GROWTH OF *Neurospora crassa*

The petridishes were marked as O (control), 1, 2, 3 and 4 for non-autoclaved and autoclaved as 0_0, 0_1, 0_2, 0_3, 0_4,with the help of a glass marking pen and the concentration were taken accordingly. 10ml of molted VM plating media was added to each petridish. The temperature of the medium should not exceed 47°C. The leaf extract and medium were mixed very well by shaking the plate gently. As for control only VM plating medium was taken in each petridish and allowed to become semi-solid. Conidia from 4 days old Em 'a' was inoculated at the centre of the petridish containing semi-solid VM plating media prepared with either plant extract or no extract. In both autoclaved and non-autoclaved leaf extracts the experiments were conducted in four replications.The petridishes were kept in the incubator at 25°C and observed the radial growth at six hours interval for six times after 18 hours post inoculation.

3.4. STUDY OF EFFECT OF EXTRACTS OF *Coccinia cordifolia* (Linn.) Cogn.

A. PREPARATION OF LEAF EXTRACTS OF *Coccinia cordifolia* (Linn.) Cogn. (TELAKUCHA)

Collected leaf samples were rinsed with running tap water followed by washing with distilled water and air dried. 50gm of fresh leaves of *Coccinia cordifolia* (Linn.) Cogn. (Telakucha) was measured with the help of a balance and crushed to fine powder in mortar pestle using liquid N_2 or keeping in ice. The paste was collected in a falcon tube and centrifuge for 10 minutes at 4°C. Then the supernatant was collected into another falcon tube. These concentration was taken in duplicate and one set was sterilized in the autoclave at 121°C under 15 Ib pressure for 20 minutes and other set was kept at 4°C incubator to use as non sterilized from.

B. PREPARATION OF DIFFERENT SOLUTION OF *Coccinia cordifolia* (Linn.) Cogn. LEAVES

Different concentrations (25, 50, 75 and 100%) of leaf extract of *Coccinia cordifolia* (Linn.) Cogn. solutions with sterilized distilled water were prepared (Table 3).

Table 3. Different concentrations of *Coccinia cordifolia* (Linn.) Cogn. extract solution.

Name of the solutions	Amount of crude extracts	Amount of sterilized distilled water	Concentration of the crude extract in the solution in %
C_1	1ml	3ml	25%
C_2	2ml	2ml	50%
C_3	3ml	1ml	75%
C_4	4ml	0ml	100%

The prepared solutions were kept separately in sterilized test tubes.

C. EVALUATION OF THE EFFECT OF NON-AUTOCLAVED (NORMAL) AND AUTOCLAVED LEAF EXTRACT OF *Coccinia cordifolia* (Linn.) Cogn. (TELAKUCHA) AGAINST THE GROWTHOF *Neurospora crassa*

The petridishes were marked as O (control), 1, 2, 3 and 4 for non-autoclaved and autoclaved as C_0, C_1, C_2, C_3, C_4, with the help of a glass marking pen and the concentration were taken accordingly. 10ml of molted VM plating media was added to each petridish. The temperature of the medium should not exceed 47°C. The leaf extract and medium were mixed very well by shaking the plate gently. As for control only VM plating medium was taken in each petridish and allowed to become semi-solid. Conidia from 4 days old Em 'a' was inoculated at the centre of the petridish containing semi-solid VM plating media prepared with either plant extract or no extract. In both autoclaved and non-autoclaved leaf extracts the experiments were conducted in four replications. The petridishes were kept in the incubator at 25°C and observed the radial growth at six hours interval for six times after 18 hours post inoculation.

3.5. STUDY OF EFFECT OF EXTRACTS OF *Andrographis paniculata* (Burm.f.) Wall.

A. PREPARATION OF LEAF EXTRACTS OF *Andrographis paniculata* (Burm.f.) Wall. (KALOMEGH)

Collected leaf samples were rinsed with running tap water followed by washing with distilled water and air dried. 50gm of fresh leaves of *Andrographis paniculata* (Burm.f.) Wall. was measured with the help of a balance and crushed to fine powder in mortar pestle using liquid

N_2 or keeping in ice. The paste was collected in a falcon tube and centrifuge for 10 minutes at 4°C. Then the supernatant was collected into another falcon tube. These concentration was taken in duplicate and one set was sterilized in the autoclave at 121°C under 15 Ib pressure for 20 minutes and other set was kept at 4°C incubator to use as non sterilized from.

B. PREPARATION OF DIFFERENT SOLUTIONS OF *Andrographis paniculata* (Burm. f.) Wall. LEAVES

Different concentrations (25, 50, 75 and 100%) of leaf extract of *Andrographis paniculata* (Burm. f.) Wall. solutions with sterilized distilled water were prepared (Table 4).

Table 4. Different concentrations of *Andrographis paniculata* (Burm. f.) Wall. extract solution.

Name of the solutions	Amount of crude extracts	Amount of sterilized distilled water	Concentration of the crude extract in in the solution in %
A_1	1ml	3ml	25%
A_2	2ml	2ml	50%
A_3	3ml	1ml	75%
A_4	4ml	0ml	100%

The prepared solutions were kept separately in sterilized test tubes.

C. EVALUATION OF THE EFFECT OF NON-AUTOCLAVED (NORMAL) AND AUTOCLAVED LEAF EXTRACT OF *Andrographis paniculata* (Burm.f.) Wall. (KALOMEGH) AGAINST THE GROWTH OF *Neurospora crassa*

The petridishes were marked as O (control), 1, 2, 3 and 4 for non-autoclaved and autoclaved as A_0, A_1, A_2, A_3, A_4, with the help of a glass marking pen and the concentration were taken accordingly. 10ml of molted VM plating media was added to each petridish. The temperature of the medium should not exceed 47°C. The leaf extract and medium were mixed very well by shaking the plate gently. As for control only VM plating medium was taken in each petridish and allowed to become semi-solid. Conidia from 4 days old Em ‘a’ was inoculated at the centre of the petridish containing semi-solid VM plating media prepared with either plant extract or no extract. In both autoclaved and non-autoclaved leaf extracts the experiments were conducted in four replications. The petridishes were kept in the incubator at 25°C and observed the radial growth at six hours interval for six times after 18 hours post inoculation.

3.6. STUDY OF EFFECT OF EXTRACTS OF *Centella asiatica* (Linn.) Urban.

A. PREPARATION OF LEAF EXTRACTS OF *Centella asiatica* (Linn.) Urban. (THANKUNI)

Collected leaf samples were rinsed with running tap water followed by washing with distilled water and air dried. 50gm of fresh leaves of *Centella asiatica* (Linn.) Urban. (Thankuni) was measured with the help of a balance and crushed to fine powder in mortar pestle using liquid N_2 or keeping in ice. The paste was collected in a falcon tube and centrifuge for 10 minutes at 4°C. Then the supernatant was collected into another falcon tube. These concentration was taken in duplicate and one set was sterilized in the autoclave at 121°C under 15 Ib pressure for 20 minutes and other set was kept at 4°C incubator to use as non sterilized from.

B. PREPARATION OF DIFFERENT SOLUTIONS OF *Centella asiatica* (Linn.) Urban. LEAVES

Different concentrations (25, 50, 75 and 100%) of leaf extract of *Centella asiatica* (Linn.) Urban. solutionswith sterilized distilled water were prepared (Table 5).

Table 5. Different concentrations of *Centella asiatica* (Linn.) Urban.extract solution.

Name of the solutions	Amount of crude extracts	Amount of sterilized distilled water	Concentration of the crude extract in the solution in %
T_1	1ml	3ml	25%
T_2	2ml	2ml	50%
T_3	3ml	1ml	75%
T_4	4ml	0ml	100%

The prepared solutions were kept separately in sterilized test tubes.

C. EVALUATION OF THE EFFECT OF NON-AUTOCLAVED (NORMAL) AND AUTOCLAVED LEAF EXTRACT OF *Centella asiatica* (Linn.) Urban. (THANKUNI) AGAINST THE GROWTH OF *Neurospora crassa*

The petridishes were marked as O (control), 1, 2, 3 and 4 for non-autoclaved and autoclaved as T_0, T_1, T_2, T_3, T_4, with the help of a glass marking pen and the concentration were taken

accordingly. 10ml of molted VM plating media was added to each petridish. The temperature of the medium should not exceed 47°C. The leaf extract and medium were mixed very well by shaking the plate gently. As for control only VM plating medium was taken in each petridish and allowed to become semi-solid. Conidia from 4 days old Em 'a' was inoculated at the centre of the petridish containing semi-solid VM plating media prepared with either plant extract or no extract. In both autoclaved and non-autoclaved leaf extracts the experiments were conducted in four replications. The petridishes were kept in the incubator at 25°C and observed the radial growth at six hours interval for six times after 18 hours post inoculation.

3.7. STUDY OF EFFECT OF EXTRACTS OF *Azadirachta indica* A. Juss.

A. PREPARATION OF LEAF EXTRACTS OF *Azadirachta indica* A. Juss. (NEEM)

Collected leaf samples were rinsed with running tap water followed by washing with distilled water and air dried. 50gm of fresh leaves of *Azadirachta indica* A. Juss. (Neem) was measured with the help of a balance and crushed to fine powder in mortar pestle using liquid N_2 or keeping in ice. The paste was collected in a falcon tube and centrifuge for 10 minutes at 4°C. Then the supernatant was collected into another falcon tube. These concentration was taken in duplicate and one set was sterilized in the autoclave at 121°C under 15 Ib pressure for 20 minutes and other set was kept at 4°C incubator to use as non sterilized from.

B. PREPARATION OF DIFFERENT SOLUTIONS OF *Azadirachta indica* A. Juss. LEAVES

Different concentrations (25, 50, 75 and 100%) of leaf extracts of *Azadirachta indica* A. Juss. solution with sterilized distilled water were prepared (Table 6).

Table 6. Different concentrations of *Azadirachta indica* A. Juss. extract solution.

Name of the solutions	Amount of crude extracts	Amount of sterilized distilled water	Concentration of the crude extract in in the solution in %
N_1	1ml	3ml	25%
N_2	2ml	2ml	50%
N_3	3ml	1ml	75%
N_4	4ml	0ml	100%

The prepared solutions were kept separately in sterilized test tubes.

C. EVALUATION OF THE EFFECT OF NON-AUTOCLAVED (NORMAL) AND AUTOCLAVED LEAF EXTRACT OF *Azadirachta indica* A. Juss. (NEEM) AGAINST THE GROWTH OF *Neurospora crassa*

The petridishes were marked as O (control), 1, 2, 3 and 4 of non-autoclaved and autoclaved as N_0, N_1, N_2, N_3, N_4, with the help of a glass marking pen and the concentration were taken accordingly. 10ml of molted VM plating media was added to each petridish. The temperature of the medium didn't exceed 47°C. The leaf extract and medium were mixed very well by shaking the plate gently. As for control only VM plating medium was taken in each petridish and allowed to become semi-solid. Conidia from 4 days old Em 'a' was inoculated at the centre of the petridish containing semi-solid VM plating media prepared with either plant extract or no extract. In both autoclaved and non-autoclaved leaf extracts the experiments were conducted in four replications. The petridishes were kept in the incubator at 25°C and observed the radial growth at six hours interval for six times after 18 hours post inoculation.

D. EVALUATION OF THE EFFECT OF NON-AUTOCLAVED (NORMAL) AND AUTOCLAVED LEAF EXTRACT OF *Azadirachta indica* A. Juss. (NEEM) AGAINST THE GROWTH OF *Helminthosporium oryzae*

The petridishes were marked as O (control), 1, 2, 3 and 4 for non-autoclaved and autoclaved as N_0, N_1, N_2, N_3, N_4, with the help of a glass marking pen and the concentration were taken accordingiy. 10ml of molted Potato Dextrose Agar (PDA) media was added to each petridish. The temperature of the medium should not exceed 47°C. The leaf extract and medium were mixed very well by shaking the plate gently. As for control only Potato Dextrose Agar (PDA) medium was taken in each petridish. When the media was cooled down then the petriplates were inoculated with mycelium disc (4mm diameter) of the test fungus taken from the margin of five days old pure culture. The mycelium disc inoculated on PDA with no plant extracts but with only sterile water acts as control plate. The radial growth of *Helminthosporium oryzae* was measured after five days of incubation. In both autoclaved and non-autoclaved leaf extracts the experiments were conducted in four replications. The whole work was done in the laminar airflow to avoid contamination. The petridishes were kept in the incubator at 25°C and observed the radial growth at six hours interval for six times after 18 hours post inoculation. This test was done only for the best plant extract.

3.8. COMPARISON OF ANTIFUNGAL ACTIVITY OF THE PLANT EXTRACTS OF ALL FIVE PLANT SAMPLES AGAINST *Neurospora crassa*

Plant extract were prepared from each plant as mentioned earlier technique (3A). 10 ml molted VM plating media was poured in the petridish when the medium cooled down to 47°Ctemperature. Five holes were made in the solidified plating media and the holes were marked as 1, 2, 3, 4, 5where each hole size was 6 mm in diameter. Each hole contained different crude extracts. 4 days old conidia of Ema was inoculated at the centre of the petridish. The experiment was conducted in three replications. The whole work was done in the laminar airflow cabinet to avoid contamination. The petridishes were kept in the incubator at 25°C and observed the radial growth at six hours interval for two times after 18 hours post inoculation.

3.9. FRACTIONATION OF PROTEINS OF *Azadirachta indica* A. juss. LEAF EXTRACT

Clean young leaves of *Azadirachta indica* A. Juss.was crushed to fine powder using liquid N_2.Then 50 ml phosphate buffered saline (PBS) was added with the leaf powder, mixed well and kept in 4°C for overnight. After centrifuge for 10 minutes at 4°C 28 ml extract were collected to a new collection tube. The extracts were collected in falcon tube and kept at 4°C.2.11 gm of ammonium sulphate (10% saturation) were mixed with 28 ml extract, stired and kept at 4°C for two hours. Centrifuged the mixture at 13000 rpm for 10 minutes at 4°C and separated the pellet and supernatant. Dissolved the pellet in 10mM tris and 100mM NaCl and this was the first fraction. The remaining soup was then used for preparing second fraction. For this, 2.11 gm of ammonium sulphate was added to the soup and kept in 4°C for another two hour after mixing by stiring. The mixture was centrifuged at 13000 rpm for 10 minutes and separated the pellet and supernatant. The pellet was dissolved in 10 mM Tris and 100mM NaCl to get fraction 2. Thus repeated up to 100% saturation and five fractions were obtained indicated as fraction 1, fraction 2, fraction 3, fraction3, fraction 4 and fraction 5 and fraction E the end product. These were kept in 4°C. All the steps were performed on Ice.

3.10. PURIFICATION OF DIFFERENT PROTEIN FRACTIONS OF *Azadirachta indica* A. Juss. LEAF EXTRACT

Ammonium sulphate was used to separate proteins present in the leaf extracts. To get pure protein in the fraction salt must be removed. Therefore Sephadex G 25 (Sigma- Aldrich Company) was used to purify the collected fractions during the study. Sephadex G25 is a fast and efficient medium for the purification of large molecules (nucleic acids, complex carbohydrates, peptides, proteins) from small molecules (nucleotides, labels and salts). 1.5 ml sephadex columns were packed in a 3 ml seringe. Then the columns were calibrated using natural gravitation force by adding PBS solution from the top. Then the plant extracts were passed through the column to get salt free extracts.This method was performed on Ice.

3.11. POLYACRYLAMIDE GEL ELECTROPHRESIS (NATIVE PAGE AND SDS PAGE) FOR PROTEIN ANALYSIS OF *Azadirachta indica* A. Juss. LEAVES

Polyacrylamide gel electrophoresis separates protein molecules present in complex mixtures according to their sizes and charges. During electrophoresis there is an intricate interaction among samples, gel matrix buffer and electric current resulting in separate bands of individual molecules. Polyacrylamide gel electrophoresis was performed in vertical mini slab gel (8 cm ×7 cm) separated by 0.75 mm thick spacer. The electrophoresis was done using the discontinuous buffer system.

Preparation of solution and reagents:

30% Acrylamide: 29.2 gm of Acrylamide and 0.8 gm of N, N' methelenebisacrylamide were dissolved in 100 ml of deionised double distilled water and stored in a dark bottle at 4°C.

Separating gel buffer (1.5 M Tris-Hcl pH 8.8): 18.16 gm of Tris was dissolved in 80 ml of deionised doubled distilled water, pH was adjusted to 8.8 with concentrated HCl and volume was made up to 100 ml with deionised doubled distilled water.

Stacking gel buffer (0.5 M Tris-HCl pH 6.8): 12.11 gm of Tris was dissolved in 80 ml of deionised doubled distilled water, pH was adjusted to 6.8 with concentrated HCl and volume was made up to 100ml with deionised doubled distilled water.

10% APS: 0.1 gm of Amonium per sulfate (APS) was dissolved in 1.0 ml of deionised doubled distilled water. APS provides the free radical which promotes the polymerization of Acrylamide and N, N' methenebisacrylamide.

TEMED: TEMED was used directly. It accelerates the polymerization of Acrylamide and N, N' mthelenebisacrylamide by catalizing the formation of free radicals from APS.The above

mentioned solutions and reagents were used in appropriate proportion to caste gel. Table 7 shows the components of the gel and their amount needed for casting of the two types of gel.

Table 7. Composition of 10 ml separating gel (12%) and 5 ml stacking gel

Solution components	Component volume for one gel mold (ml)	Component volume for one gel mold (ml)
	Separating gel (12%)for 12ml	Stacking gel for 5 ml
Deionised doubled distilled water	3.2	2.975
0.5 M Tris-HCl, pH 6.8	-	1.25
1.5 M Tris-HCl, pH 8.8	2.6	
30% Acrylamide	4	0.67
10% ammonium persulphate (AP)	0.1	0.05
TEMED	0.01	0.005

1× Running Buffer (Lower electrode buffer)

25mM Tris-HCl

200mM glycine

0.1% (w/v) SDS

(Approximately vol. of less than 1 liter is needed depending on the type electrophoresis system.)

5×Sample Buffer (Loading Buffer)

20% w/v Glycerol

0.2M Tris-HCl, pH 6.8

0.05% w/v Bromophenolblue

After adding all the constituents of separating gel, the entire mixture was immediately poured between the glass plates leaving an appropriate gap for the stacking gel (about 3 cm) from the top of the glass plate. Then deionised doubled distilled water was carefully overlidded on the

separating geland kept undisturbed for several minutes allowing the gel to polymerize. After removing the overlaying water layer, all the constituents of stacking gel was mixed and immediately poured on the polymerized separating gel, a telfon comb was inserted in to the poured stacking gel. It was then kept undisturbed for half an hour for polymerization.

Before loading the samples were diluted in a ratio of 1:1 with sample buffer and to denature the coiled proteins the sample protein along with loading buffer was heated at 95°C for five minutes.

After polymerization of the gel, combs were removed from the gel plates. The gel holdings tank was filled with Running Buffer and the casted gel should be dipped in Buffer.

Power conditions:

Power to the mini-PROTEAN Tetra cell was supplied by an external DC Voltage power supply and the electrophoresis was allowed to start 120V constant is recommended for native gel applications. 120 V was applied approximately for 45 minutes.

3.12. ESTIMATION OF SOLUBLE PROTEIN IN *Azadirachta indica* A. Juss. LEAVES

Soluble protein in *Azadirachta indica* A. Juss. leaf extracts were estimated by the method of Lowry *et al*. (1951) using bovine serum albumin as standard.

Preparation of samples:

Reagent -1: Reagent 1 was prepared by mixing the following solutions-

i) 49 ml of 1% NaOH + 1% anhydrous Na_2CO_3

ii) 0.5 ml of 1% Cu SO_4. $5H_2O$

iii) 0.5ml of 2% Na-K –tertrate

Reagent-2

Folin and ciocalteu's phenol diluted to 1:1 with H_2O

Procedure:

i) 20 µl of sample protein was taken in test tube.

ii) 980 µl of sterilized distilled water was added.

iii) 5 ml of reagent-1 was added in each tube.

iv) Allowed 5 minutes to occur the reaction.

v) 0.5 ml reagent- 2 was added in each test tube and mixed properly.

vi) Kept for 30 minutes in dark till the colour developed.

vii) Measured the optical density (OD) at 750 nm.

Estimation of protein: Optical density (OD) was measured by spectrophotometer at 750 nm. Amount of protein in various samples were estimated from the standard curve following the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard.

3.13. ANTIFUNGAL ACTIVITY ANANLYSIS USING FRACTIONATED PROTEIN

The petridishes were marked as F_O (control plate), F_1, F_2, F_3, F_4, F_5 & F_E with the help of a glass marking pen. 10ml of molted media (VM, PDA) was poured to each petridish and 200µg of protein was added from each fraction into the designated plates. The temperature of the medium should not exceed 47°C. The protein fractions and medium were mixed very well by shaking the plate gently. Only the normal growing medium (VM, PDA) for specific fungi was used for control. When the media was solidified conidia from 4 days old (*Neurospora crassa* and *Helminthosporium oryzae*) were inoculated at the centre of the petridish. All the experiemts were conducted in four replications. The whole work was done in the laminar flow cabinet to avoid contamination. The petridishes were kept in the incubator at 25°C and observed after 24 hours.

3.14. ANTIFUNGAL ACTIVITY ANANLYSIS USING SPECFIC PROTEIN

The petridishes were marked as A, B, C and D with the help of a glass marking pen and the same size protein bands were cut and directly used with growth media (VM and PDA). 10ml of molted media (VM, PDA) was added to each petridish. The temperature of the medium should not exceed 47°C. The protein bands and medium were mixed very well by shaking the plate gently. Only the normal growing medium (VM, PDA) for specific fungi was used for control. When the media was solidified conidia from 4 days old (*Neurospora crassa* and *Helminthosporium oryzae*) were inoculated at the centre of the petridish. All the experiemts were conducted in two replications. The whole work was done in the laminar airflow cabinet

to avoid contamination. The petridishes were kept in the incubator at 25°C and observed after 24 hours.

RESULTS

4.1. Effects of non-autoclaved and autoclaved leaf extracts of *Ocimum sanctum* Linn. on the growth of *Neurospora crassa*

For testing the effect *Ocimum sanctum* Linn. four different concentration were taken separately on the sterilized petridishes with the help of sterilized pipette. The petridishes were marked as 0 (no extract only VM), 1 (25% extract), 2 (50% extract), 3 (75% extracts), 4 (100% extracts) for non-autoclaved (normal) and autoclaved as O_0 (no extract only VM), O_1 (25% extract), O_2 (50% extract), O_3 (75% extract), O_4 (100% extract) where 10 ml molten VM was added in each petridish. When media became solid Em'a' was inoculated at the centre of the media. Plates were incubated at 25ºC and measured the radial growth at six hours intervalafter 18 hours post inoculation.

It was observed that, the radial growth of wild type *Neurospora crassa* was controlled by different concentrations of *Ocimum sanctum* Linn. leaf extracts. The radial growth of *Neurospora crassa* was observed for 48 hours at 6 hours intervals. Non-autoclaved 25% crude extract (plate no.1) showed 1.33 cm, 2.06 cm, 2.76 cm, 3.83 cm, 4.33 cm growth over radial growth. 50% crude extract (plate no. 2) showed 0.9 cm, 1.53 cm, 2.1 cm, 2.73 cm, 3.6 cm and 4.2 cm radial growth. 75% crude extract (plate no. 3) showed 0.63 cm, 1.06 cm, 1.63 cm, 2.03 cm, 2.8 cm and 3.73 cm radial growth. 100% crude extract (plate no. 4) showed 0.43 cm, 0.83 cm, 1.23 cm, 1.73 cm, 2.5 cm and 3.06 cm radial growth. These data were presented in table 8. Whereas, autoclaved 25% crude extract (plate no.O_1) showed 1.46 cm, 2.4 cm, 2.93 cm, 3.83 cm, 4.6 cm and growth over radial growth. 50% crude extract (plate no.O_2) showed 1.06 cm, 1.76 cm, 2.5 cm, 3.1 cm, 3.9 cm and 4.3 cm radial growth. 75% crude extract (plate no.O_3) showed 0.9 cm, 1.43 cm, 2.03 cm, 2.4 cm, 3.03 cm and 3.8 cm radial growth. 100% crude extract (plate no.O_4) showed 0.5 cm, 0.96 cm, 1.9 cm, 2.4 cm, 2.8 cm and 3.5 cm radial growth at 18, 24, 30, 36, 42 and 48 hours, respectively. Control plate (no extract only VM) presented 1.73 cm, 2.57 cm, 3.46 cm, 4.3 cm, growth over radial growth. These data were presented in table 9.Based on the radial growth size, it can be concluded that 100% concentrated non-autoclaved plant extracts were more effective than its autoclaved counterparts (Figure 9). The size of radial growth was 0.83cm and 0.96 cm after 24 hours incase of non-autoclaved and autoclaved plant extracts, respectively. Although with

extended time period, radial growth was increased but comparative study revealed that non-autoclaved plant extracts were better at 100% concentration than the control and other concentrations (Figure 10). It was also revealed that with increased concentration of plant extract, fungal growth was prohibited. Instead of having antifungal properties, leaf extract of the *Ocimum sanctum* Linn. was unable to restrict the growth of *Neurospora crassa* completely (Figure 9).

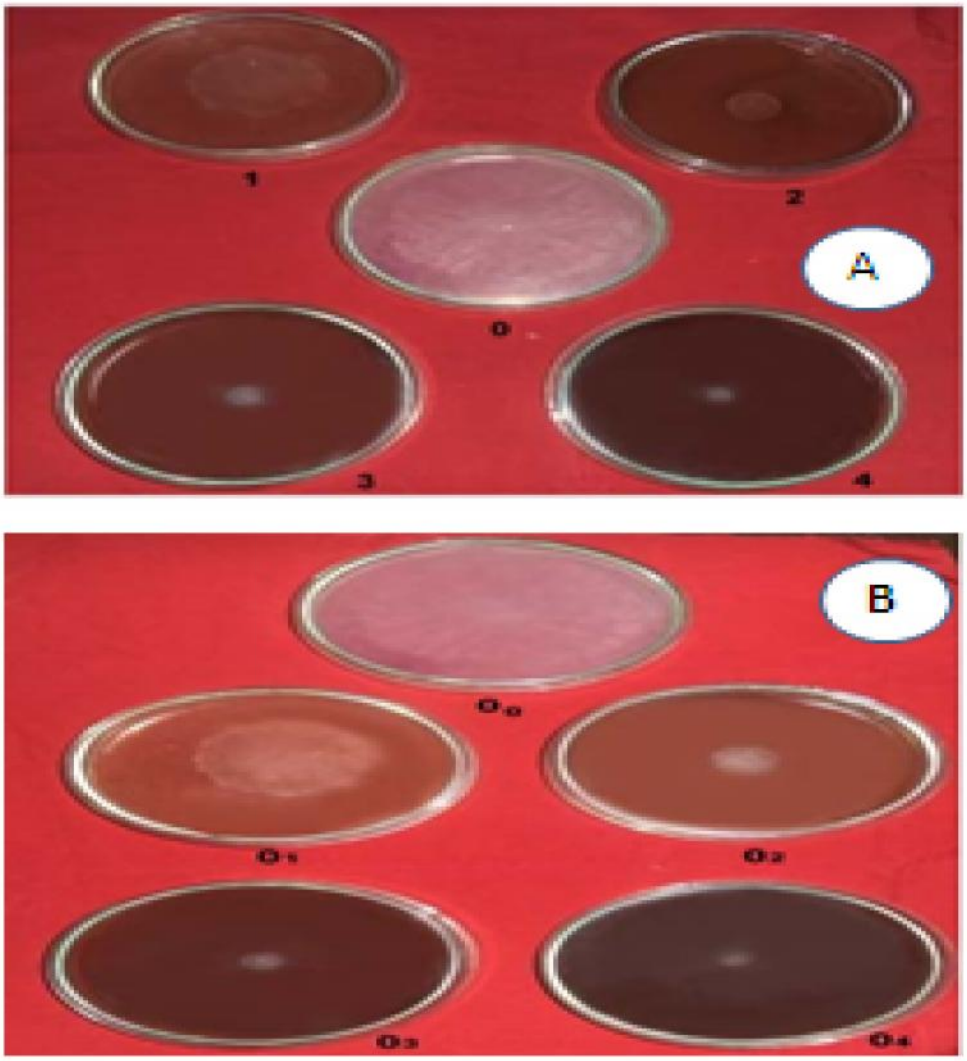


Figure 9. Comparative radial growth of *Neurospora crassa* on VM containing different concentrations of non-autoclaved (A) and autoclaved (B) leaf extracts of *Ocimum sanctum* Linn. (after 24 hours) where 0= control plate, 1= 25% crude extract, 2= 50% crude extract,

3= 75% crude extract, 4= 100% crude extract for non-autoclaved leaf extracts and where 0_0= control plate, 0_1= 25% crude extract, 0_2= 50% crude extract, 0_3= 75% crude extract, 0_4= 100% crude extract for autoclaved leaf extracts.

Table 8. **Radial growth of Em 'a' on VM containing non-autoclaved (normal) leaf extracts of *Ocimum sanctum* Linn.**

Plate and concentration of the solution	Radial growth in cm at different time points					
	18 hours	24 hours	30 hours	36 hours	42 hours	48 hours
0 (no extract only VM)	1.73	2.5	3.46	4.3	Over Gr.	-
1 (25% crude extract)	1.33	2.06	2.76	3.83	4.33	Over Gr.
2 (50% crude extract)	0.9	1.53	2.1	2.73	3.6	4.2
3 (75% crude extract)	0.63	1.06	1.63	2.03	2.8	3.73
4 (100% crud extract)	0.43	0.83	1.23	1.73	2.5	3.06

Over Gr. - Over growth

Table 9. Radial growth of Em 'a' on VM containing autoclaved leaf extracts of *Ocimum sanctum* Linn.

Plate and concentration of the solution	Radial growth in cm at different time points					
	18 hours	24 hours	30 hours	36 hours	42 hours	48 hours
0_0 (no extract only VM)	1.73	2.57	3.46	4.3	Over Gr.	-
0_1 (25% crude extract)	1.46	2.4	2.93	3.83	4.6	Over Gr.
0_2 (50% crude extract)	1.06	1.76	2.5	3.1	3.9	4.3
0_3 (75% crude extract)	0.9	1.43	2.03	2.4	3.03	3.8
0_4 (100% crude extract)	0.5	0.96	1.96	1.96	2.8	3.5

Over Gr. - Over growth

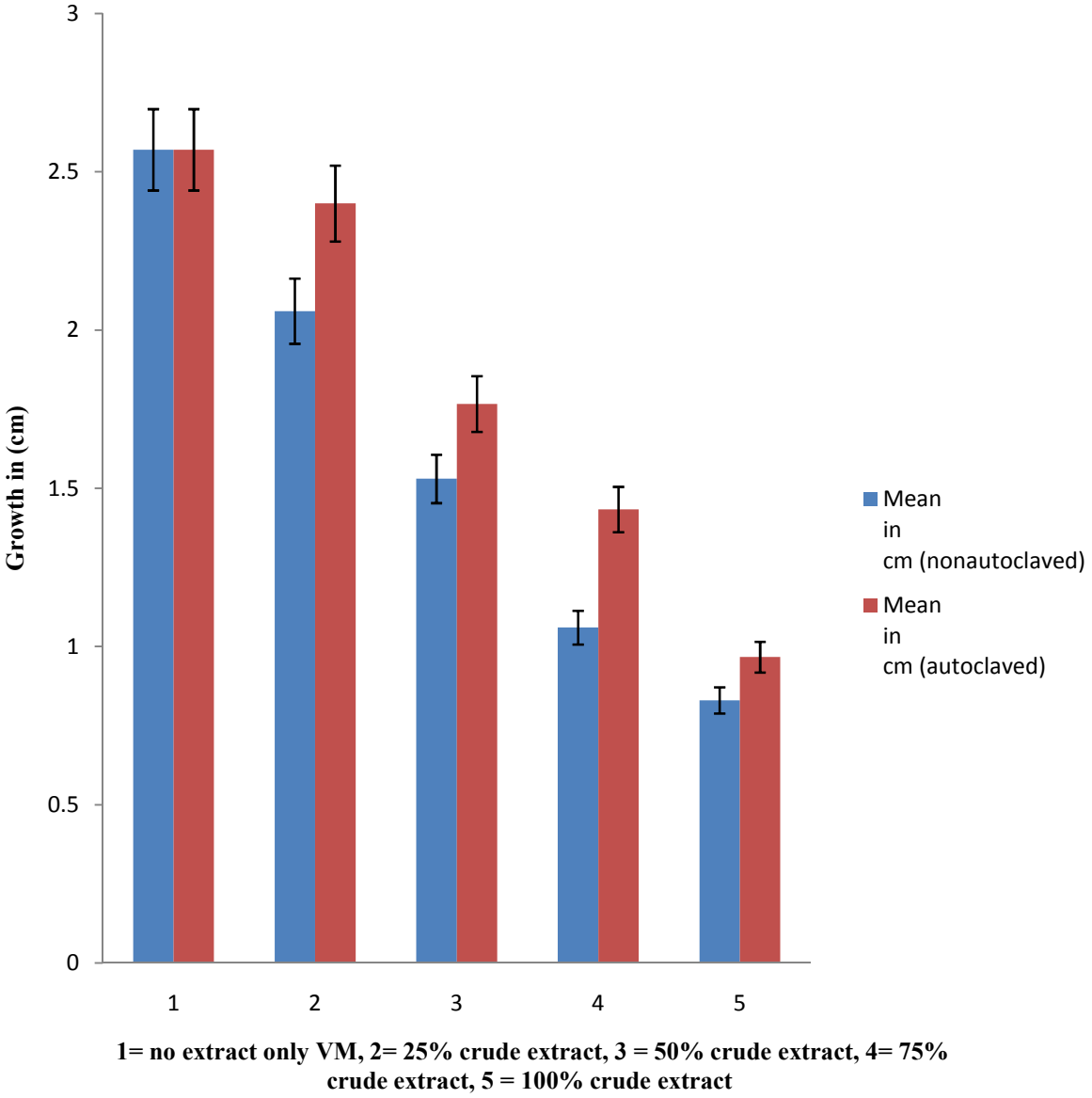


Figure 10. Graph showing radial growth of *Neurospora crassa* on VM plates containing non-autoclaved (normal) or autoclaved leaf extracts of *Ocimum sanctum* Linn. (after 24 hours).

4.2. Effects of non-autoclaved and autoclaved leaf extracts of *Coccinia cordifolia* (Linn.) Cogn. on the growth of *Neurospora crassa*

To test the antifungal effect of *Coccinia cordifolia* (Linn.) Cogn. four different concentration were taken separately on the sterilized petridishes with the help of sterilized pipette. The petridishes were marked as 0 (no extract only VM), 1(25% extract), 2 (50% extract), 3 (75% extracts), 4 (100% extracts) of normal (non-autoclaved) and autoclaved C_0 (no extract only VM), C_1 (25% extract), C_2 (50% extract), C_3 (75% extract), C_4 (100% extract) and 10 ml molten VM was added in each petridish. When media became solid Em 'a' was inoculated at the centre of the media. Plates were incubated at 25ºC and after18 hours of post inoculation the radial growth was measured at six hours interval.

It was observed that, the concentration of leaf extract of *Coccinia cordifolia* (Linn.) Cogn. controlled the radial growth of wild type *Neurospora crassa.* After 18 hours of post inoculation, the radial growth of *Neurospora crassa* was observed for 48 hours with 6 hours of time interval. Non-autoclaved 25% crude extract (plate no.1) showed 1.73 cm, 2.57 cm, 3.46 cm, 4.3 cm growth over radial growth. 50% crude extract (plate no.2) showed 1.3cm, 1.9 cm, 2.26 cm, 2.86 cm, 3.5 cm and 4.13 cm radial growth. 75% crude extract (plate no.3) showed 1 cm, 1.73 cm, 2.03 cm, 2.5 cm, 2.96 cm and 3.83 cm radial growth. 100% crude extract (plate no. 4) showed 0.8 cm, 1.33 cm, 1.9 cm, 2.46 cm, 3.0 cm and 3.8 cm radial growth. These data were presented in table 10. On the otherhand, autoclaved 25% crude extract (plate no. C_1) showed 1.80 cm, 2.36cm, 2.93 cm, 3.7 cm, 4.2 cm growth over radial growth. 50% crude extract (plate no. C_2) showed 1.4 cm, 2.03 cm, 2.73 cm, 3.3 cm, 4.0 cm, and 4.36 cm radial growth. 75% crude extract (plate no. C_3) showed 01.2 cm, 1.86 cm, 2.26 cm, 2.9 cm, 3.73 cm and 4.3 cm radial growth. 100% crude extract (plate no. C_4) showed 0.93 cm, 1.53 cm, 2.03 cm, 2.7 cm, 3.2 cm and 4.1 cm radial growth at 18, 24, 30, 36, 42 and 48 hours, respectively. Control plate (no extract only VM) showed 1.8 cm, 2.53 cm, 3.56 cm ,4.5 cm and growth over radial growth. The data were summarized in table 11.

It was observed from the result that non-autoclaved concentrations were more effective than its autoclaved counterparts (Figure 11). Where non- autoclaved 4 no. plate showed 1.33 cm radial growth and autoclaved O_4 plate had 1.53 cm radial growth at 24 hours. Though with increased time period, radial growth was also increased (incase of non-autoclaved 4 no. plate concentration produced 3.8 cm radial growth at 48 hours) but comparative study revealed that non-autoclaved (plate no.4) was better than the control plate and other concentrations (Figure

12). It was also revealed that with the increasing concentration of plant extracts, fungal growth was prohibited. Similar result was also observed in case of *Coccinia cordifolia* (Linn.) Cogn. where leaf extracts showed antifungal properties, but the growth of *Neurospora crassa* growth was not completely restricted (Fig.11).

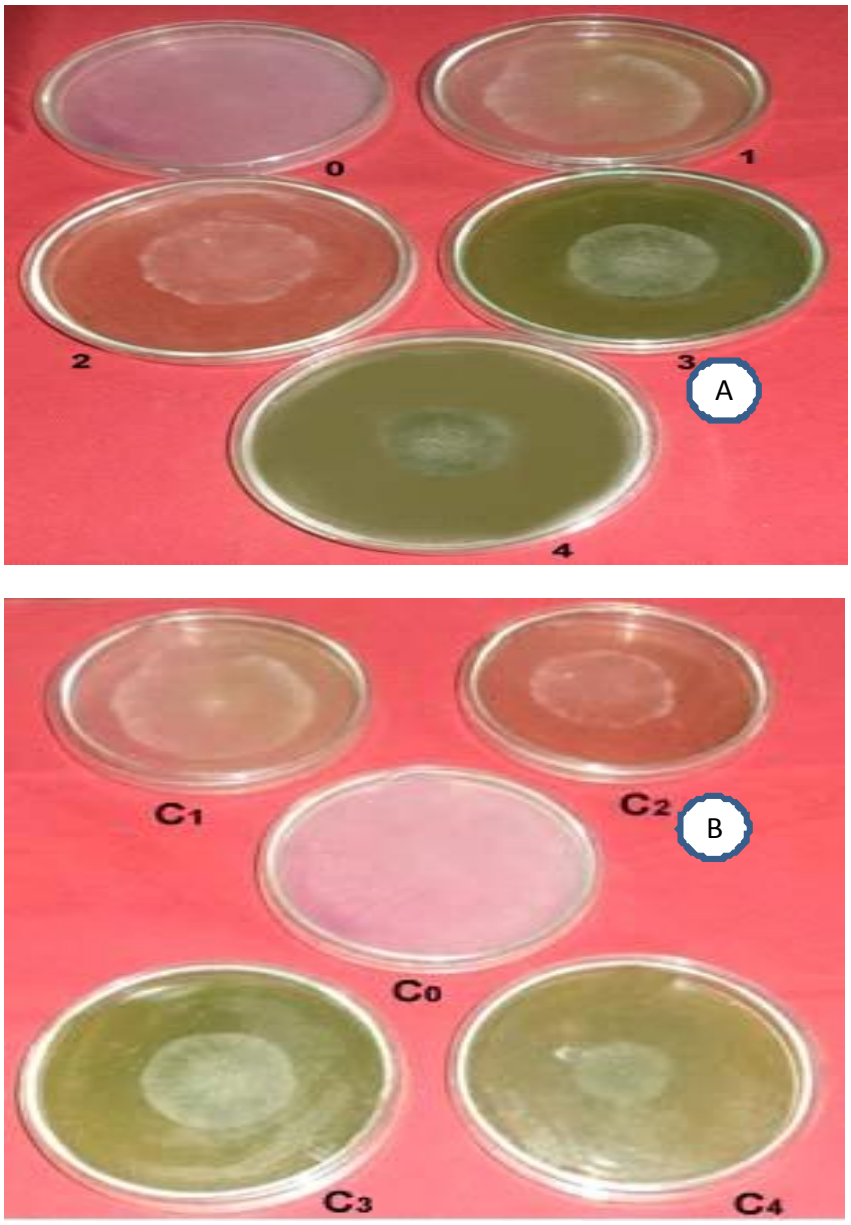


Figure11. Comparative radial growth of *Neurospora crassa* on VM containing different concentrations of non-autoclaved (A) and autoclaved (B) leaf extracts of *Coccinia cordifolia* (Linn.) Cogn. (after 24 hours) where 0= control plate, 1= 25% crude extract, 2= 50% crude extract, 3= 75% crude extract, 4= 100% crude extract. C_0= control plate, C_1= 25% crude extract, C_2= 50% crude extract, C_3= 75% crude extract, C_4= 100% crude extract.

Table 10. Radial growth of Em 'a' on VM containing non-autoclaved (normal) leaf extracts of *Coccinia cordifolia* (Linn.) Cogn.

Plate and concentration of the solution	Radial growth in cm at different time points					
	18 hours	24 hours	30 hours	36 hours	42 hours	48 hours
0 (no extract only VM)	1.73	2.57	3.46	4.3	Over Gr.	-
1 (25% crude extract)	1.73	2.57	3.46	4.3	Over Gr.	-
2 (50% crude extract)	1.3	1.9	2.26	2.86	3.5	4.13
3 (75% crude extract)	1.0	1.73	2.03	2.5	3.0	3.83
4 (100% crude extract)	0.8	1.33	1.9	2.46	2.9	3.8

Over Gr. - Over growth

Table 11. Radial growth of Em 'a' on VM containing autoclaved leaf extracts of *Coccinia cordifolia* (Linn.) Cogn.

Plate and concentration of the solution	Radial growth in cm at different time points					
	18 hours	24 hours	30 hours	36 hours	42 hours	48 hours
C_0 (no extract only VM)	1.8	2.53	3.56	4.4	Over Gr.	-
C_1 (25% crude extract)	1.8	2.36	2.93	3.7	4.2	Over Gr.
C_2 (50% crude extract)	1.4	2.03	2.73	3.3	4.0	4.36
C_3 (75% crude extract)	1.2	1.86	2.26	2.9	3.73	4.3
C_4 (100% crude extract)	0.93	1.53	2.03	2.7	3.2	4.1

Over Gr. - Over growth

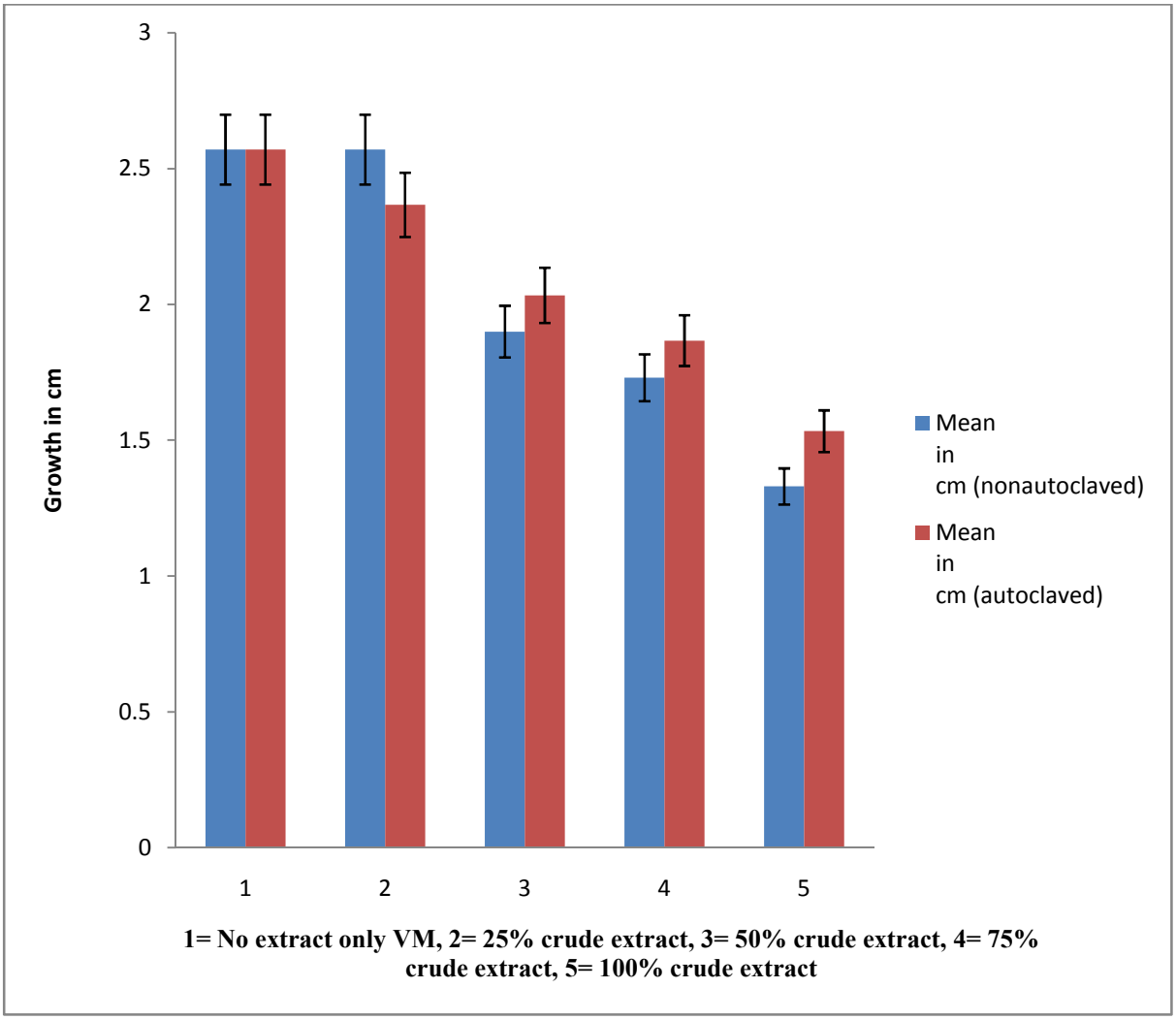


Figure 12. Graph showing radial growth of *Neurospora crassa* on VM plates containing non-autoclaved (normal) or autoclaved leaf extracts of *Coccinia cordifolia* (Linn.) Cogn. (after 24 hours)

4.3. Effects of non-autoclaved and autoclaved leaf extracts of *Andrographis paniculata* (Burm. f.) Wall. on the growth of *Neurospora crassa*

To test the antifungal effect of *Andrographis paniculata*, different solutions were taken separately on the sterilized petridishes with the help of sterilized pipette. The petridishes were marked as 0 (no extract only VM), 1 (25% extract), 2 (50% extract), 3 (75% extracts), 4 (100% extracts) of normal (non-autoclaved) and autoclaved A_0 (no extract only VM), A_1 (25% extract), A_2 (50% extract), A_3 (75% extract), A_4 (100% extract) and 10 ml molten VM was added in each petridish. When media became solid Em 'a' was inoculated at the centre of the media. Plates were incubated at 25ºC and measured the radial growth at six hours interval after18 hours post inoculation.

It was revealed that, leaf extract of *Andrographis paniculata* (Burm. f.) Wall. was able to control the radial growth of wild type *Neurospora crassa* at different concentrations. The radial growth of *Neurospora crassa* was observed for 48 hours at 6 hours intervals. Non-autoclaved 25% crude extract (plate no.1) showed 1.33 cm, 2.06 cm, 2.76 cm, 3.83 cm, 4.33 cm and growth over radial growth. 50% crude extract (plate no.2) showed 0.9 cm, 1.53 cm, 2.1 cm, 2.73 cm, 3.6 cm and 4.2 cm radial growth. 75% crude extract (plate no.3) showed 10.63 cm, 1.06 cm, 1.63 cm, 2.03 cm, 2.8 cm and 3.73 cm radial growth. 100% crude extract (plate no. 4) showed 0.43 cm, 0.83 cm, 1.23 cm, 1.73 cm, 2.5 cm and 3.06 cm radial growth. These data were presented in table 12. Whereas, autoclaved 25% crude extract (plate no. A_1) showed 1.46 cm, 2.4 cm, 2.93 cm, 3.83 cm, 4.6 cm and growth over radial growth. 50% crude extract (plate no. A_2) showed 1.06 cm, 1.76 cm, 2.5 cm, 3.1 cm, 3.9 cm and 4.36 cm radial growth. 75% crude extract (plate no. A_3) presented 0.9 cm, 1.43 cm, 2.03 cm, 2.4 cm, 3.03 cm and 3.8 cm radial growth. 100% crude extract (plate no. A_4) showed 0.5 cm, 0.96 cm, 1.96 cm, 2.4 cm, 2.8 cm, 3.5 cm radial growth at 18, 24, 30, 36, 42 and 48 hours, respectively. Control plate (no extract only VM) showed 1.73 cm, 2.57 cm, 3.46 cm and 4.3 cm growth over radial growth. The data were summarized in table 13.

Based on the radial growth size, it can be concluded that 100% concentrated non-autoclaved plant extracts were more effective than its autoclaved counterparts (Figure 13). The size of radial growth was 0.83 cm and 0.96 cm at 24 hours incase of non-autoclaved and autoclaved plant extracts, respectively. Although with extended time period, radial growth was increased but comparative study revealed that non-autoclaved plant extracts were better at 100% concentration than the control and other concentrations (Figure 14). It was also revealed that with increased concentration of plant extracts, fungal growth was prohibited. Instead of

having antifungal properties, leaf extracts of the *Andrographis paniculata* (Burm. f.) Wall. was unable to restrict the growth of *Neurospora crassa* completely (Figure 13).

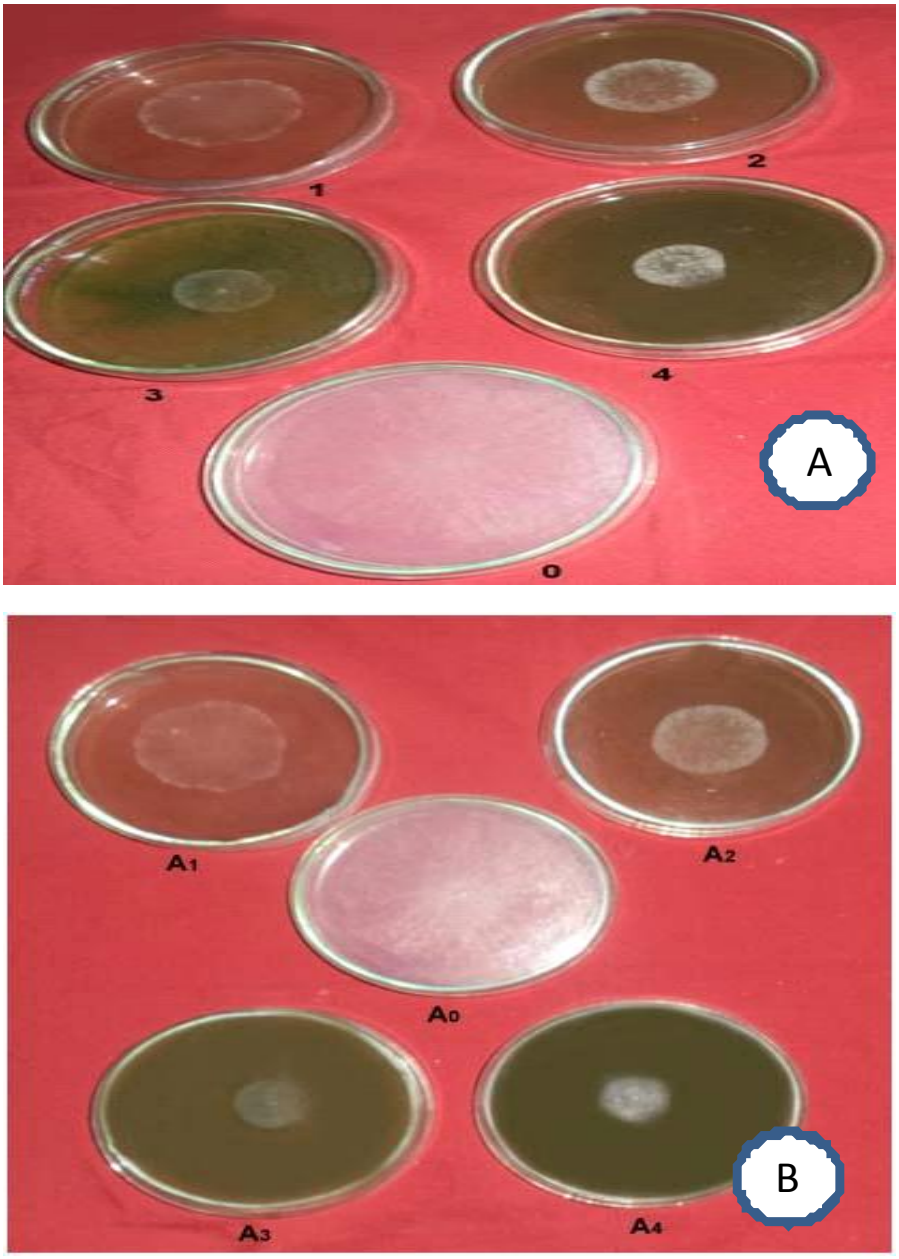


Figure 13. Comparative radial growth of *Neurospora crassa* on VM containing different concentrations of non-autoclaved (A) and autoclaved (B) leaf extracts of *Andrographis paniculata* (Burm. f.) Wall. (after 24 hours) where 0= control plate, 1= 25% crude extract, 2= 50% crude extract, 3= 75% crude extract, 4= 100% crude extract. A_0= control plate, A_1= 25% crude extract, A_2= 50% crude extract, A_3= 75% crude extract, A_4= 100% crude extract.

Table 12. Radial growth of Em 'a' on VM containing non-autoclaved (normal) leaf extracts of *Andrographis paniculata* (Burm. f.) Wall.

Plate and concentration of the solution	Radial growth in cm at different time points					
	18 hours	24 hours	30 hours	36 hours	42 hours	48 hours
0 (no extract only VM)	1.73	2.57	3.46	4.3	Gr. over	-
1 (25% crude extract)	1.33	2.06	2.76	3.83	4.33	Gr. over
2 (50% crude extract)	0.9	1.53	2.10	2.73	3.6	4.2
3 (75% crude extract)	0.63	1.06	1.63	2.03	2.8	3.73
4 (100% crude extract)	0.43	0.83	1.23	1.73	2.5	3.06

Over gr.= over growth

Table 13. Radial growth of Em'a' on VM containing autoclaved leaf extracts of *Andrographis paniculata* (Burm. f.) Wall.

Plate and concentration of the solution	Radial growth in cm at different time points					
	18 hours	24 hours	30 hours	36 hours	42 hours	48 hours
A_0 (no extract only VM)	1.73	2.57	3.46	4.3	Over Gr.	-
A_1 (25% crude extract)	1.46	2.4	2.93	3.8	4.4	Over Gr.
A_2 (50% crude extract)	1.06	1.76	2.5	3.1	3.9	4.3
A_3 (75% crude extract)	0.9	1.43	2.03	2.4	3.1	3.73
A_4 (100% crude extract)	0.5	0.96	1.96	1.96	2.8	3.5

Over gr.= Over growth

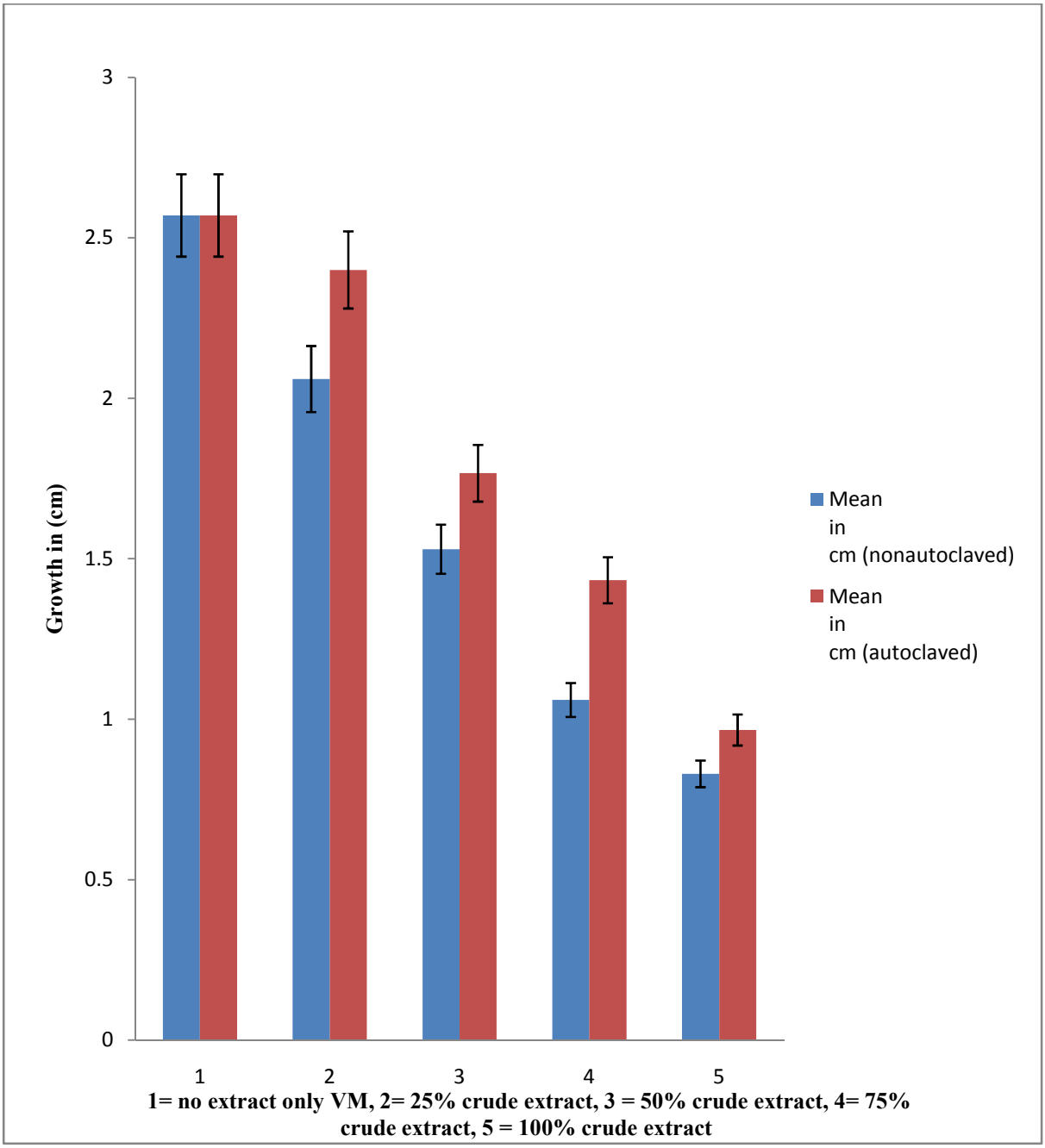


Figure 14. Graph showing radial growth of *Neurospora crassa* on VM plates containing non-autoclaved (normal) or autoclaved leaf extracts of *Andrographis paniculata* (Burm. f.) Wall. (after 24 hours).

4.4. Effects of non-autoclaved and autoclaved leaf extract of *Centella asiatica* (Linn.) Urban. on the growth of *Neurospora crassa*

For testing the antifungal effect of *Centella asiatica*, different solutions were taken separately on the sterilized petridishes with the help of sterilized pipette. The petridishes were marked as 0 (no extract only VM), 1 (25% extract), 2 (50% extract), 3 (75% extract), 4 (100% extract) of normal (non-autoclaved) and autoclaved T_0 (no extract only VM), T_1 (25% extract), T_2 (50% extract), T_3 (75% extract), T_4 (100% extract) and 10 ml molten VM was added in each petridish. When media solidified, Em 'a' was inoculated at the centre of the media. Plates were incubated at 25ºC and measured the radial growth at six hours interval after18 hours post inoculation.

It was observed that, the concentration of leaf extract of *Centella asiatica* (Linn.) Urban. controlled the radial growth of wild type *Neurospora crassa.* After 18 hours of post inoculation, the radial growth of *Neurospora crassa* was observed for 48 hours at 6 hours of time interval. Non-autoclaved 25% crude extract (plate no.1) showed 1.73 cm, 2.63 cm, 3.2 cm, 3.8 cm, 4.2 cm and over growth radial growth. 50% crude extract (plate no.2) showed 1.23 cm, 1.9 cm, 2.46 cm, 3.1 cm, 3.8 cm and 4.3 cm radial growth. 75% crude extract (plate no.3) showed 0.93cm, 1.83 cm, 2.3 cm, 2.9 cm, 3.5 cm and 3.96 cm radial growth. 100% crude extract (plate no.4) showed 0.8 cm, 1.26 cm, 1.76 cm, 2.23 cm, 2.76 cm and 3.8 cm radial growth. The data were summarized in table 14. Whereas, autoclaved 25% crude extract (plate no. T_1) showed 1.86 cm, 2.3 cm, 3.3 cm, 3.9cm, 4.5 cm, and over growth radial growth. 50% crude extract (plate no. T_2) showed 1.43 cm, 2.03 cm, 2.7 cm, 3.2 cm, 3.9 cm, and 4.53cm radial growth. 75% crude extract (plate no. T_3) showed 1.16 cm, 1.9 cm, 2.53 cm, 3.1 cm, 3.8 cm and 4.3 cm radial growth. 100% crude extract (plate no. T_4) showed 0.9 cm, 1.43 cm, 1.96 cm, 2.46 cm, 2.8 cm, 3.9 cm radial growth at 18, 24, 30, 36, 42 and 48 hours, respectively. Control plate (no extract only VM) showed 1.73 cm, 2.57 cm, 3.46 cm and 4.3 cm and growth over radial growth. These data were presented in table 15.

It was observed from the result that non-autoclaved concentrations were more effective than its autoclaved counterparts (Figure 15).Where non-autoclaved 4 no. plate showed 1.26 cm radial growth and autoclaved T_4 plate showed 1.43cm radial growth at 24 hours. Though with increased time period, radial growth was increased (incase of non-autoclaved plate no. 4 concentration produced 3.8cm radial growth at 48 hours) but comparative study revealed that non-autoclaved plate no. 4 was better than the control plate and other concentrations (Figure 16). It was also revealed that with the increasing concentration of extracts, fungal growth was

prohibited. Similar result was observed in case of *Centella asiatica* (Linn.) Urban. where leaf extracts showed antifungal properties, but the growth of *Neurospora crassa* growth was not restricted properly (Fig.15).

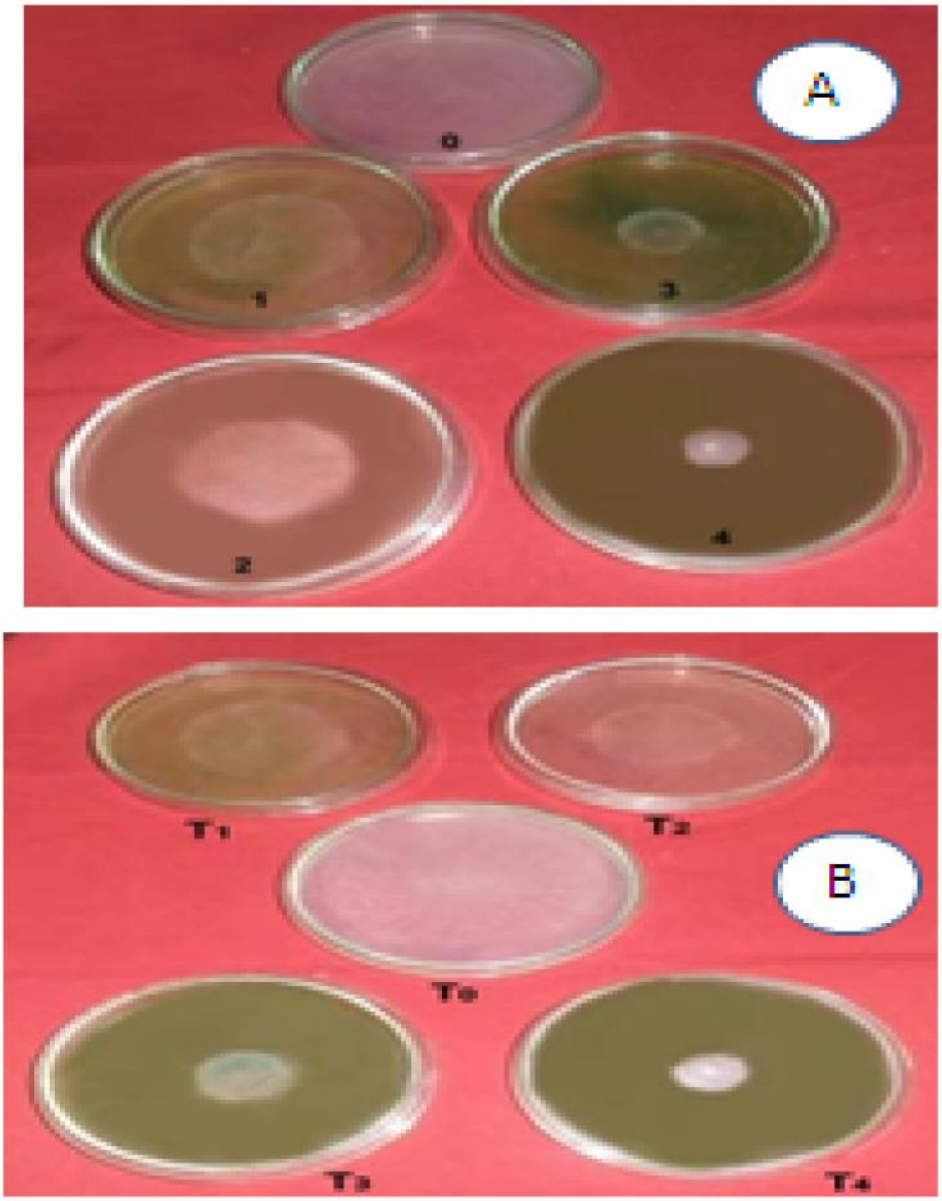


Figure 15. Comparative radial growth of *Neurospora crassa* on VM containing different concentrations of non-autoclaved (A) and autoclaved (B) leaf extracts of *Centella asiatica* (Linn.) Urban. (after 24 hours) where 0= control plate, 1= 25 % crude extract, 2= 50% crude extract, 3= 75% crude extract, 4= 100% crude extract. T_0= control plate, T_1= 25% crude extract, T_2= 50% crude extract, T_3= 75% crude extract, T_4= 100% crude extract.

Table 14. Radial growth of Em 'a' on VM containing non-autoclaved (normal) leaf extracts of *Centella asiatica* (Linn.) Urban.

Plate and concentration of the solution	Radial growth in cm at different time points					
	18 hours	24 hours	30 hours	36 hours	42 hours	48 hours
0 (no extract only VM)	2.0	2.8	3.4	4.0	4.5	Over Gr.
1 (25% crude extract)	1.73	2.67	3.2	3.8	4.2	Over Gr.
2 (50% crude extract)	1.23	1.9	2.46	3.1	3.8	4.3
3 (75% crude extract)	0.96	1.83	2.3	2.9	3.5	3.96
4 (100% crude extract)	0.8	1.26	1.76	2.26	2.76	3.8

Over Gr. = Over growth

Table 15. Radial growth of Em 'a' on VM containing autoclaved leaf extracts of *Centella asiatica* (Linn.) Urban.

Plate and concentration of the solution	Radial growth in cm at different time points					
	18 hours	24 hours	30 hours	36 hours	42 hours	48 hours
T_0 (no extract only VM)	2.0	2.8	3.4	4.0	4.5	Over Gr.
T_1 (25% crude extract)	1.86	2.8	3.3	3.9	4.4	Over Gr.
T_2 (50% crude extract)	1.43	2.03	2.7	3.2	3.9	4.53
T_3 (75% crude extract)	1.2	1.9	2.53	3.1	3.8	4.3
T_4 (100% crude extract)	0.9	1.43	1.96	2.46	2.8	3.9

Over Gr.= Over growth

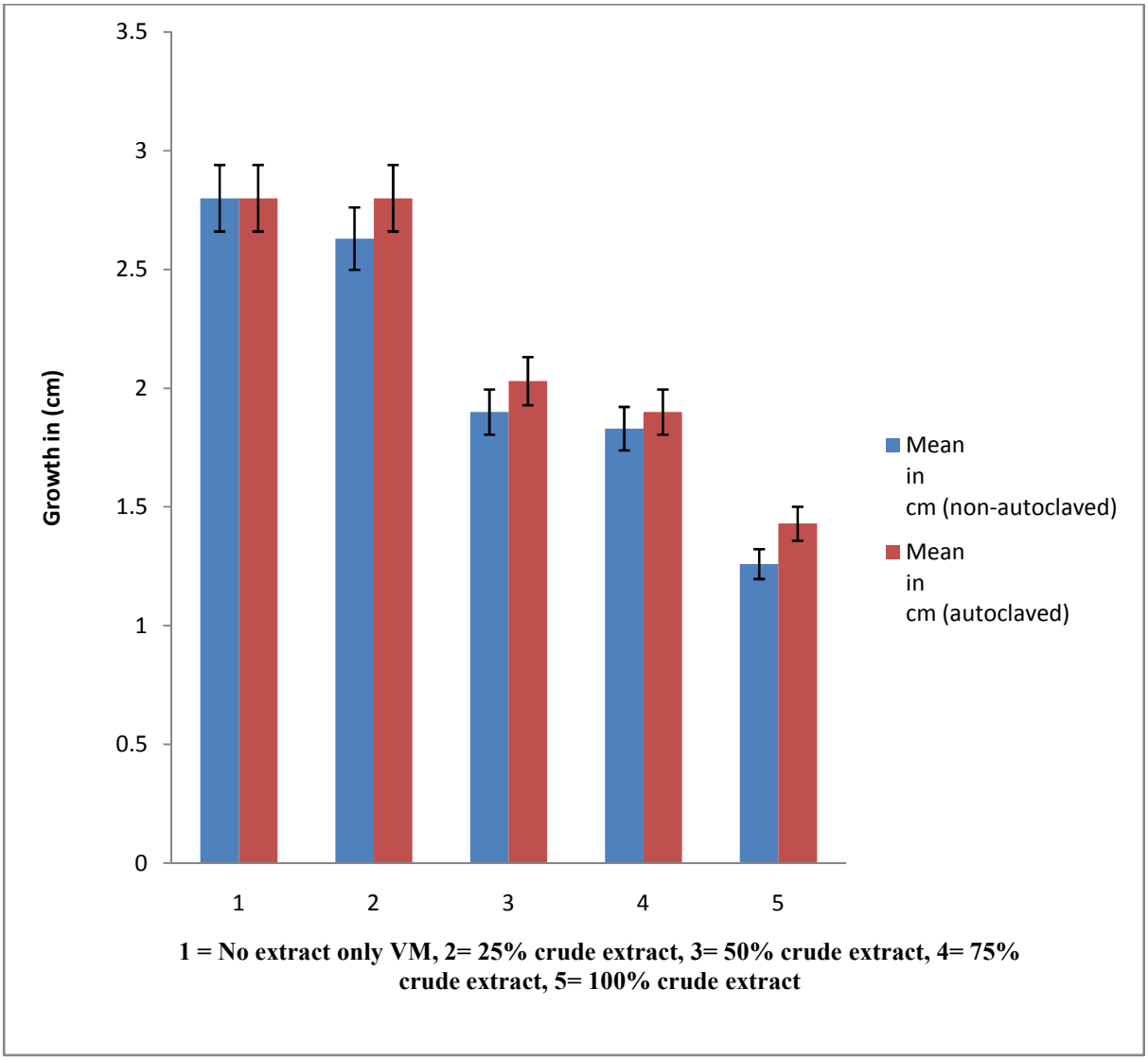


Figure 16. Graph showing radial growth of *Neurospora crassa* on VM plates containing non-autoclaved (normal) or autoclaved leaf extracts of *Centella asiatica* (Linn.) Urban. (after 24 hours)

4.5. Effects of non-autoclaved and autoclaved leaf extract of *Azadirachta indica* A. Juss. on the growth of *Neurospora crassa*

For testing the effect, different solutions were taken separately on the sterilized petridishes with the help of sterilized pipette. The petridishes were marked as 0 (no extract only VM), 1(25% extract), 2 (50% extract), 3 (75% extract), 4 (100% extract) of normal (non-autoclaved) and autoclaved N_0 (no extract only VM), N_1 (25% extract), N_2 (50% extract), N_3 (75% extract), N_4 (100% extract) and 10 ml molten VM was added in each petridish. When media became solid Em "a" was inoculated at the centre of the media. Plates were incubated at 25°C and measured the radial growth at six hours interval after18 hours post inoculation.

It was observed that, the radial growth of wild type *Neurospora crassa* was controlled by different concentrations of *Azadirachta indica* A. Juss. leaf extracts. The radial growth of *Neurospora crassa* was observed for 48 hours at 6 hours of time interval. Non- autoclaved 25% crude extract (plate no.1) showed 0.3 cm, 0.8 cm, 1.23 cm, 1.6 cm, 2.03 cm and 2.26 cm radial growth respectively. 50% crude extract (plate no.2) showed no radial growth at 18 and 24 hours, whereas it showed 0.2 cm at 30 hours, 0.46 cm at 36 hours, 0.9 cm at 42 hours and 1.36 cm radial growth at 48 hours. 75% crude extract (plate no.3) and 100% crude extract (plate no. 4) showed no radial growth at different time periods. The data were summarized table 16. Whereas, autoclaved 25% crude extract (plate no. N_1) showed 0.5 cm, 1.0 cm, 1.46 cm, 1.9cm, 2.13 cm and 2.6 cm radial growth. 50% crude extract (plate no. N_2) showed 0.2 cm, 0.6 cm, 0.96 cm, 1.3 cm, 1.66 cm and 2.2 cm radial growth. 75% crude extract (plate no. N_3) showed no growth at 18 and 24 hours,whereas 0.26 cm radial growth at 30 hours, 0.5 cm radial growth at 36 hours, 0.8 cm radial growth at 42 hours and 1.3 cm radial growth at 48 hours. 100% crude extract (plate no. N_4) showed no radial growth at 18, 24, 30, 36, 42 and 48 hours. Control plate (no extract only VM) showed 1.8 cm, 2.53 cm, 3.56 cm and 4.5 cm and growth over radial growth. The data were summarized in table 17.

Based on the radial growth size, it can be concluded that 100% concentrated non-autoclaved leaf extracts were more effective than its autoclaved counterparts (Figure 17). Where non-autoclaved plate no. 3 had no radial growth and autoclaved N_3 plate had 1.3 cm radial growth after 48 hours. Though extended time period, radial growth was increased but comparative study revealed that non-autoclaved plate no. 4 was better than the control plate and other concentrations (Figure 18). It was also revealed that with the increased concentration of extracts, fungal growth was prohibited. So, it can be concluded that 100% concentrated leaf

extracts of *Azadirachta indica* A. Juss. showed antifungal properties with complete growth inhibition of *Neurospora crassa* (Figure 17).

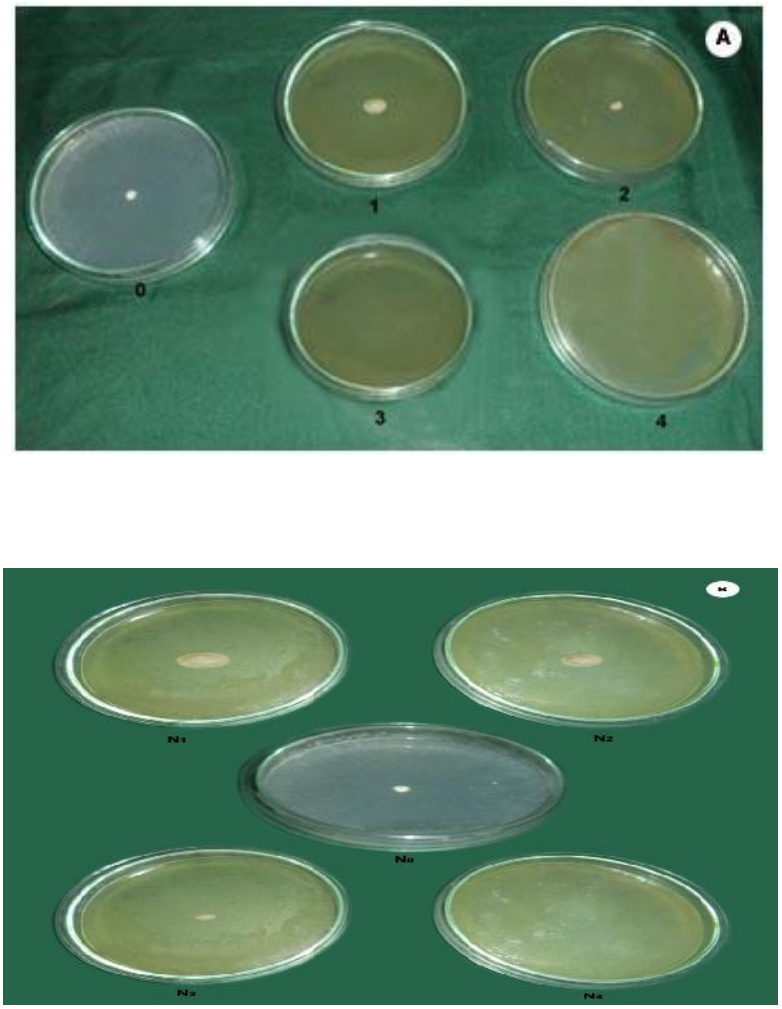


Figure 17. Comparative radial growth of *Neurospora crassa* on VM containing different concentrations of non-autoclaved (A) and autoclaved (B) leaf extracts of*Azadirachta indica* A. Juss.(after 24 hours) where 0= control plate, 1= 25% crude extract, 2= 50% crude extract, 3= 75% crude extract, 4= 100 % crude extract. N_0= control plate, N_1= 25% crude extract, N_2= 50% crude extract, N_3= 75% crude extract, N_4= 100% crude extract.

Table 16. Radial growth of Em 'a' on VM containing *Azadirachta indica* A. Juss. non-autoclaved (normal) leaf extracts

Plate and concentration of the solution	Radial growth in cm at different time points					
	18 hours	24 hours	30 hours	36 hours	42 hours	48 hours
0 (no extract only VM)	1.8	2.53	3.5	4.5	Over Gr.	-
1 (25% crude extract)	0.3	0.8	1.23	1.69	2.03	2.5
2 (50% crude extract)	No gr.	No gr.	0.2	0.5	0.9	1.36
3 (75% crude extract)	No gr.	No gr.	No gr.	No gr.	No gr.	No gr.
4 (100% crude extract)	No gr.	No gr.	No gr.	No gr.	No gr	No gr.

No Gr =No growth, Over Gr. = Over growth

Table 17. Radial growth of Em 'a' on VM containing autoclaved leaf extracts of *Azadirachta indica* A. Juss.

Plate and concentration of the solution	Radial growth in cm at different time points					
	18 hours	24 hours	30 hours	36 hours	42 hours	48 hours
N_0 (no extract only VM)	2.0	2.5	3.56	4.5	Over Gr.	-
N_1 (25% crude extract)	0.5	1.0	1.46	1.9	2.13	2.5
N_2 (50% crude extract)	0.2	0.6	0.9	1.3	1.66	2.2
N_3 (75% crude extract)	No gr.	No gr.	0.2	0.5	0.8	1.3
N_4 (100% crude extract)	No gr.	No gr.	No gr.	No gr.	No gr.	No gr.

No Gr =No growth, Over Gr. = Over growth

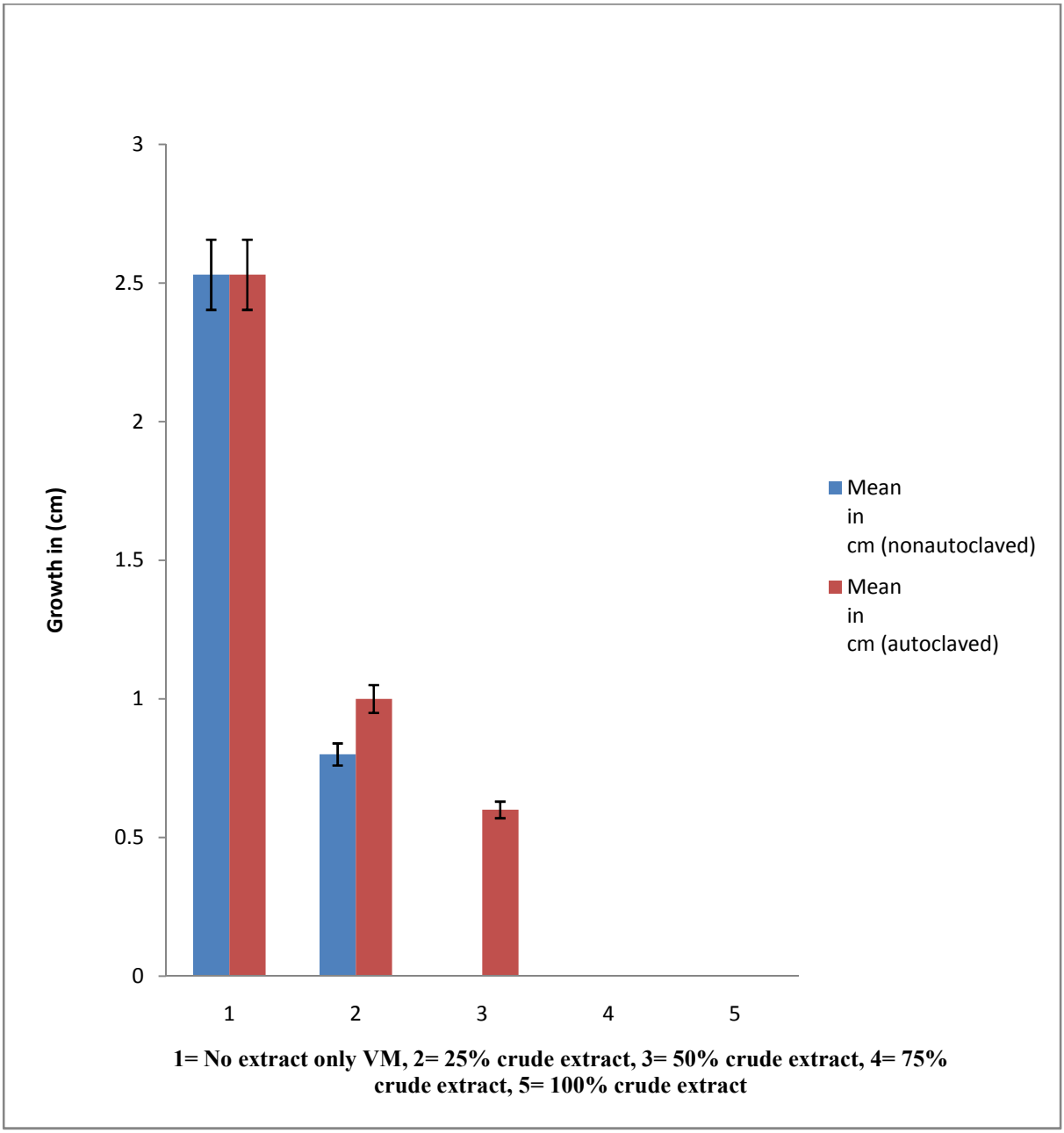


Figure18. Graph showing radial growth of *Neurospora crassa* on VM containing non-autoclaved (normal) or autoclaved leaf extracts of *Azadirachta indica* A. Juss. (after 24 hours)

4.6. Effects of non-autoclaved and autoclaved leaf extract of *Azadidrachta indica* A. juss. on the growth of *Helminthosporium oryzae*

To verify the effects of *Azadidrachta indica* A. juss. leaf extract against a pathogenic fungus *Helminthosporium oryzae*, different solutions were taken separately on the sterilized petridishes with the help of sterilized pipette. The petridishes were marked as 0 (no extract only PDA), 1(25% extract), 2 (50% extract), 3 (75% extract), 4 (100% extract) of normal (non-autoclaved) and autoclaved N_0 (no extract only), N_1 (25% extract), N_2 (50% extract), N_3 (75% extract), N_4 (100% extract) and 10 ml molten PDA was added in each petridish. When media solidified, the petriplates were inoculated with mycelium disc (4mm diameter) of the test fungus taken from the margin of five days old pure culture. The mycelium disc inoculated on PDA with no plant extracts but with only sterile water acts as control plate. The whole set up was incubated in inverted position at 26 ± 1°C incubator for 5 days. The radial growth of *Helminsthosporium oryzae* was measured after 24, 48, 72, 96 and 120 hours.

It was observed that, the concentration of leaf extract of *Azadirachta indica* A. Juss. controlled the radial growth of *Helminthosporium oryzae*. After 24 hours of post inoculation, the radial growth of *Helminthosporium oryzae* was observed for 120 hours at 12 hours of time interval. Non-autoclaved 25% crude extract (plate no. 1) showed 0.3 cm, 0.66 cm, 1.03 cm, 1.43 cm and 1.96 cm radial growth. 50% crude extract (plate no.2) showed 0.1 cm, 0.5 cm, 0.9 cm, 1.33 cm and 1.8 cm radial growth. 75% crude extract (plate no.3) and 100% crude extract (plate no. 4) showed no radial growth at different time periods. These data were presented in table 18. On the otherhand, autoclaved 25% crude extract (plate no. N_1) showed 0.5 cm, 0.8 cm, 1.2 cm, 1.6 cm and 2.03 cm radial growth. 50% crude extract (plate no. N_2) showed 0.26 cm, 0.6 cm, 1.0 cm, 1.33 cm, 1.8 cm radial growth. 75% crude extract (plate no. N_3) showed no growth at 24 hours, but it showed 0.2 cm radial growth at 48 hours, 0.5 cm radial growth at 72 hours, 0.8 cm radial growth at 96 hours and 1.1 cm radial growth at 120 hours. 100% crude extract (plate no. N_4) showed no radial growth at 24, 48, 72, 96 and 120 hours. Control plate (no extract only PDA) showed 1.5 cm, 2.8 cm, 3.76 cm, 4.23 cm and 4.7 cm radial growth. These data were presented in table 19.

It was observed from the result that non-autoclaved concentrations were more effective than its autoclaved counterparts (Figure 19). Where non-autoclaved plate no.3 showed no radial growth and autoclaved N_3 plate showed 1.1 cm radial growth at 120 hours. Though with increased time period radial growth was also increased (incase of non-autoclaved plate no.3

and 4 concentration produced no radial growth at 120 hours) but comparative study revealed that non-autoclaved plates (plate no. 3 and 4) was better than the control plate and other concentrations (Figure 20). It was also revealed that with the increasing concentration of extracts fungal growth was prohibited. Extract of the *Azadirachta indica* A. Juss. leaf showed antifungal properties, so it restricted the growth of *Helminsthosporium oryzae* (Figure 19).

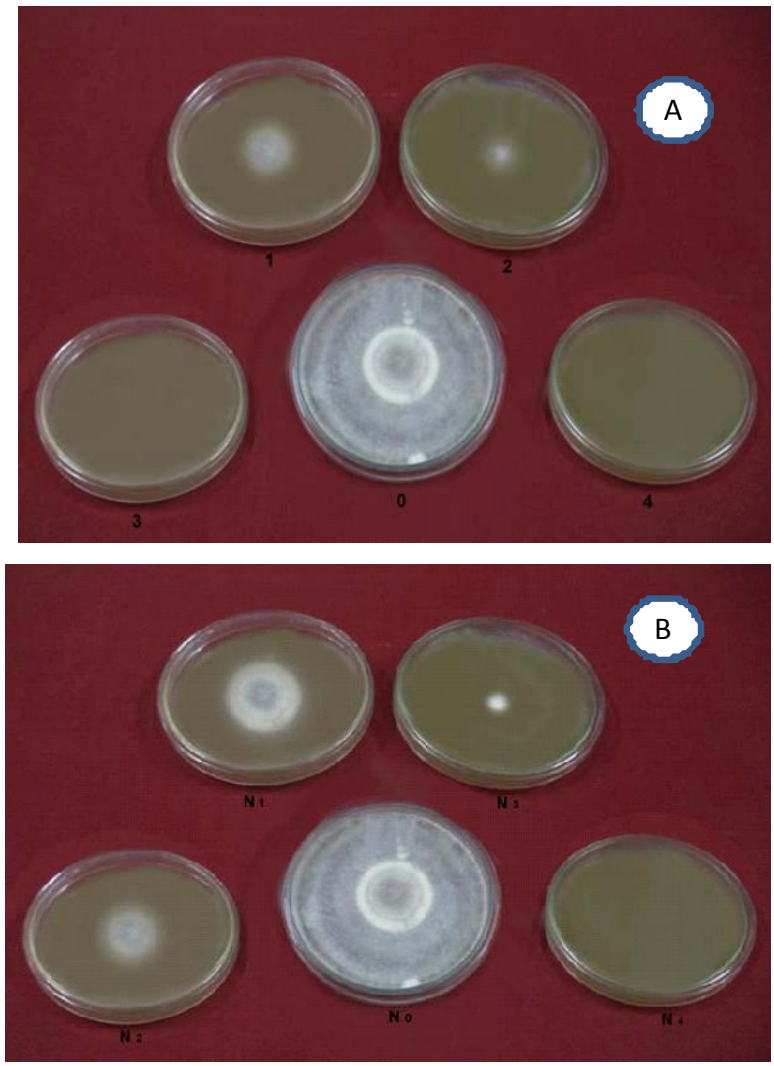


Figure 19.Comparative radial growth of *Helminthosporium oryzae* on PDA containing different concentrations of non-autoclaved (A) and autoclaved (B) leaf extracts of *Azadirachta indica* A. Juss. (after 120 hours).Where 0= control plate, 1= 25% crude extract, 2= 50% crude extract, 3= 75% crude extract, 4= 100 % crude extract. N_0= control plate, N_1= 25% crude extract, N_2= 50% crude extract, N_3= 75% crude extract, N_4= 100% crude extract.

Table 18. Radial growth of PDA containing *Azadirachta indica* A. Juss. non-autoclaved (normal) leaf extracts

Plate and concentration of the solution	Radial growth in cm at different time points				
	24 hours	48 hours	72 hours	96 hours	120 hours
0 (no extract only PDA)	1.5	2.8	3.76	4.23	4.7
1 (25% crude extract)	0.3	0.66	1.03	1.43	1.96
2 (50% crude extract)	0.1	0.5	0.9	1.33	1.8
3 (75% crude extract)	No gr.	-	-	-	-
4 (100% crude extract)	No gr.	-.	-	-	-

No gr. = No growth

Table 19. Radial growth of PDA containing *Azadirachta indica* A. Juss. autoclaved leaf extracts

Plate and concentration of the solution	Radial growth in cm at different time points				
	24 hours	48 hours	72 hours	96 hours	120 hours
N_0 (no extract only PDA)	1.5	2.8	3.76	4.23	4.7
N_1 (25% crude extract)	0.5	0.8	1.2	1.6	1.96
N_2 (50% crude extract)	0.26	0.6	1.0	1.33	1.8
N_3 (75% crude extract)	No gr.	0.2	0.5	0.8	1.1
N_4 (100% crude extract)	No gr.	-.	-	-	-

No gr. = No growth

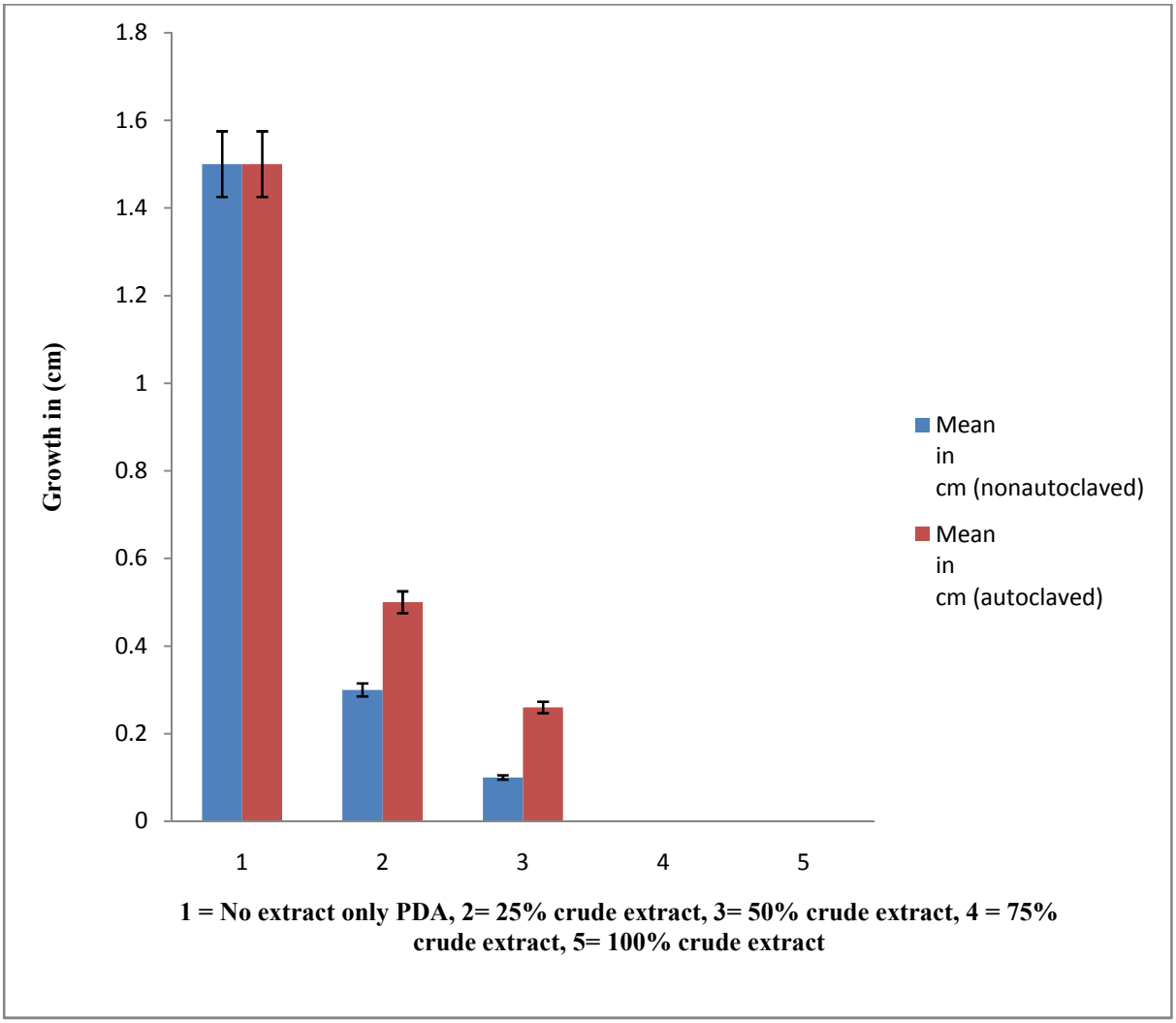


Figure 20. Graph showing radial growth of *Helminthosporium oryzae* on PDA containing non-autoclaved (normal) or autoclaved leaf extracts of *Azadirachta indica* A. Juss. (after 24 hours)

4.7. Evaluation of *Ocimum sanctum* Linn., *Coccinia cordifolia* (Linn) Cogn., *Andrographis paniculata* (Burm. f.) Wall., *Centella asiatica* (Linn.) Urban. and *Andrographis paniculata* A. Juss. against *Neurospora crassa*

To test the antifungal effects of *Ocimum sanctum* Linn., *Coccinia cordifolia* (Linn) Cogn., *Andrographis paniculata* (Burm. f.) Wall., *Centella asiatica* (Linn.) Urban. and *Andrographis paniculata* A. Juss. against *Neurospora crassa*, leaf extracts of above mentioned plants were taken separately on the same sterilized petridish with the help of sterilized pipette (400µl/ hole). The hole was cut 6 mm in size. The extracts were marked as 1 (*Ocimum sanctum* leaf extract), 2 (*Andrographis paniculata* leaf extract), 3 (*Azadirachta indica* leaf extract), 4 (*Coccinia cordifolia* leaf extract) and 5 (*Centella asiatica* leaf extracts). 10 ml molten VM was added on the petridish. When media was solidified, Em'a' was inoculated at the centre of the media. Inoculated plate was incubated at 25°C and the radial growth was measured after 24 hours. Result was shown in figure 21.

Interestingly, *Ocimum sanctum, Andrographis paniculata* and *Azadirachta indica* crude extracts were able to inhibit the radial growth of *Neurospora crassa* efficiently as compared to the *Coccinia cordifolia* and *Centella asiatica*. To be precise, *Azadirachta indica* leaf extract was found to be more competent among all the plants tested. Therefore, further study was carried out only with *Azadirachta indica* to find out its specific protein activity.

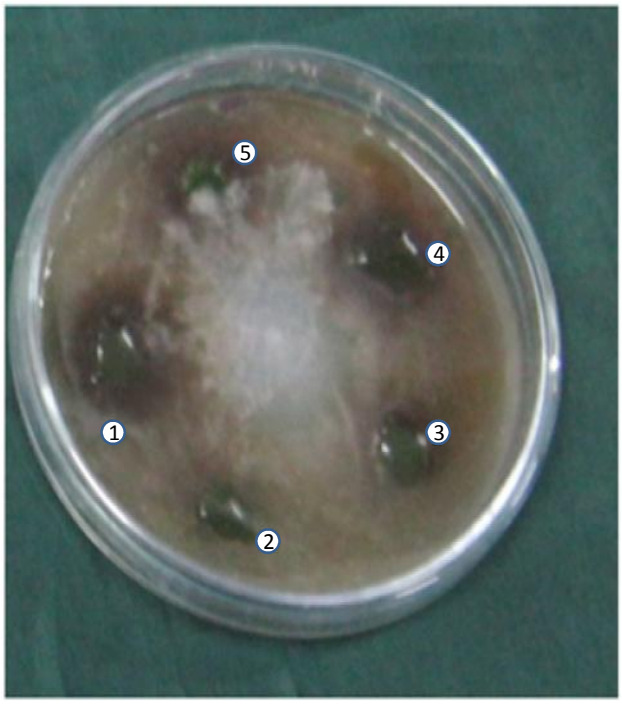


Figure 21. Effects of different leaf extracts of *Ocimum sanctum, Coccinia cordifolia*, *Andrographis paniculata, Centella asiatica, Azadirachta indica,* where 1=*Ocimum sanctum* Linn. 2=*Andrographis paniculata* (Burm. f.) Wall, 3=*Azadirachta indica* A. Juss, 4 = *Centella asiatic*a (Linn.) Urban. and 5= *Coccina cordifolia* (Linn.) Cogn.

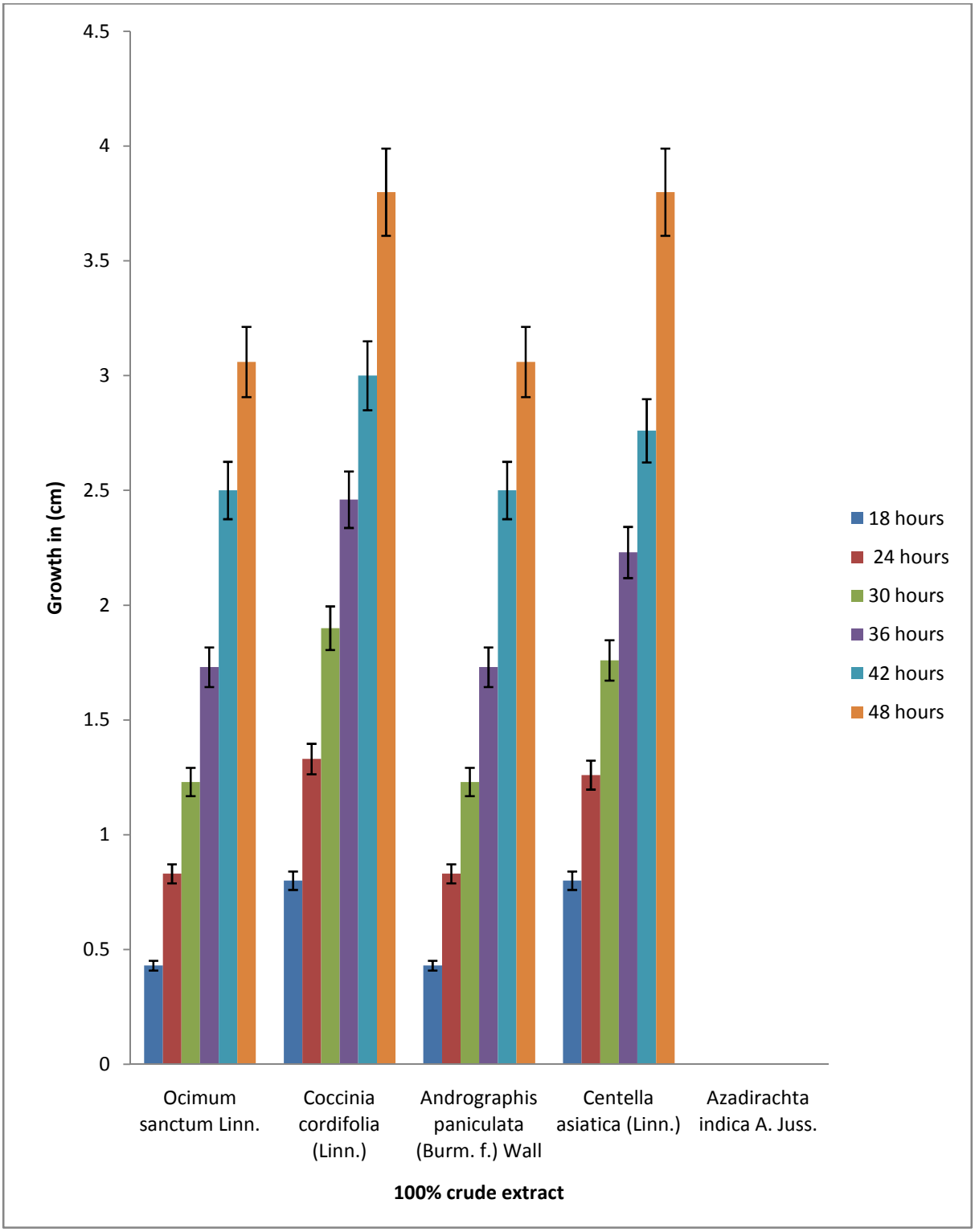
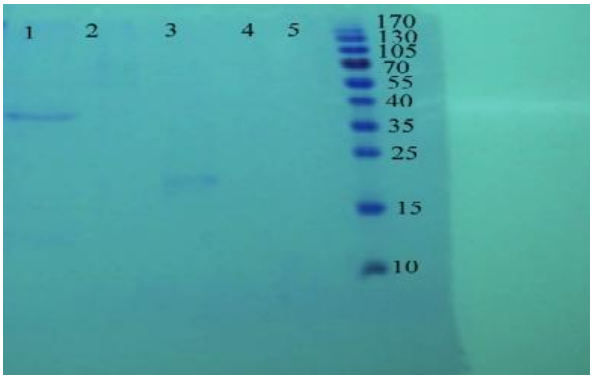


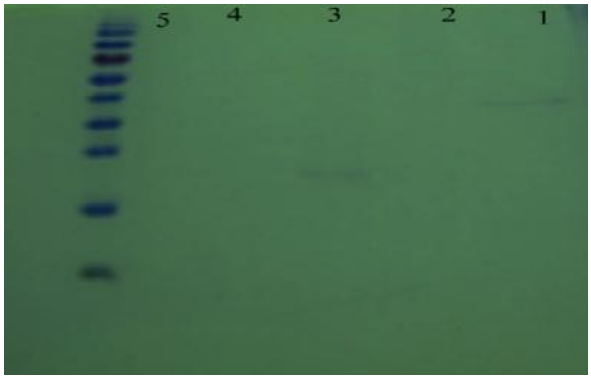
Figure 22. Graph indicating radial growth of *Neurospora crassa* with non-autoclaved leaf extracts (100% crude extract) of *Ocimum sanctum, Coccinia cordifolia*, *Andrographis paniculata, Centella asiatica, Azadirachta indica* at different time periods.

4.8. Proteomic study of leaf extracts of *Azadirachta indica* A. juss. to identify antifungal activity

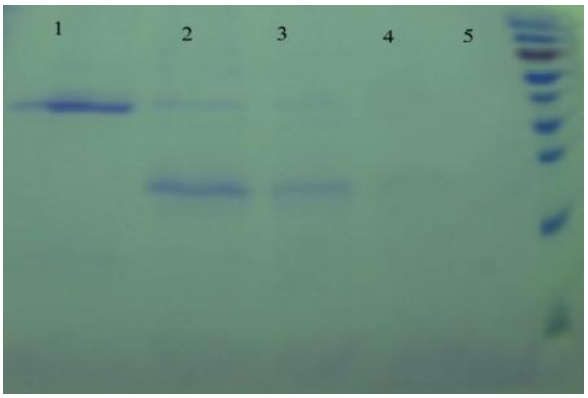
It was observed that extracts from *Azadirachta indica* inhibited the radial growth of *Neurospora crassa* more efficiently as compared to *Ocimum sanctum, Andrographis paniculata, Cocciniacordifolia* and *Centella asiatica* leaf extracts. To identify the active ingredients responsible for antifungal activity, protein were separated from *Azadirachta indica* A. juss. leaf (method no 10). Five different fractions were separated from the protein using $(NH_4)_2SO_4$ gradient centrifugation and remaining soup (end product F_E) was also collected after 100% satuaration. Five fractions and end product (F_E) were purified by Sephadex G 25 to desalt ammonium sulphate (method no 11). During purification, five elutes were collected per fraction (F_1, F_2, F_3, F_4, F_5, F_E) and protein activity of all the fractions were examined by SDS-PAGE (Figure 23). The protein activity was determined depending on the band intensity namely very dark, dark, light and very light. The darker region indicated higher amount of protein content than the lighter one. From polyacrylamide gel electrophoresis, two significant proteins bands were observed in all the five different fractions. Figure showed that three elutes per fractions (F_1, F_2, F_3, F_4, F_5) contained sufficient amount of protein whereas protein was absent in case of F_E. Fraction3 (F_3) had prominent dark band that indicated the presence of high amount of protein content as compared to other fractions. For further study, protein fractions were pooled down from first three fractions and used as total protein content.



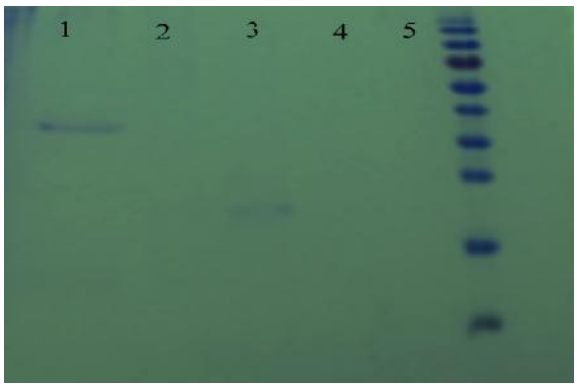
23(a)



23(b)



23 (c)



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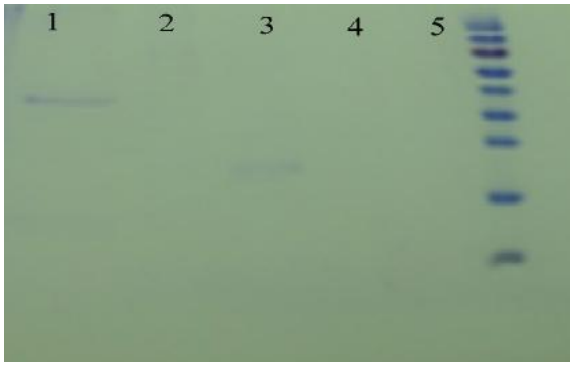
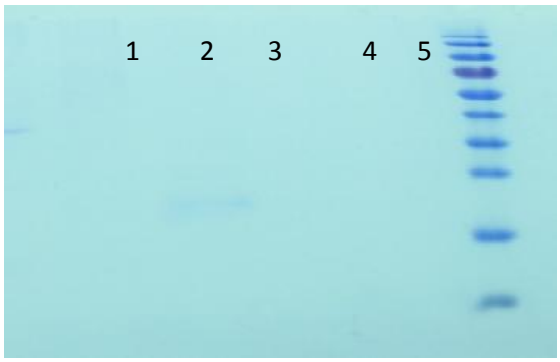


23 (e)



23 (f)

Figure 23. Different fractions of total proteins were separated in SDS-PAGE analysis. Individual fractions were eluted five times during desalted and indicated as 1, 2, 3, 4, 5 in case of each fractions such as F_1, F_2, F_3, F_4, F_5 and $F_{E.}$

4.8.1. Estimation of soluble protein in different fractions of *Azadirachta indica* A.juss.

Soluble proteins were estimated from different fractions of *Azadirachta indica* A. Juss. leaves according to Lowry *et al.* (1951) using Bovine Serum Albumin as standard. The optical density (OD) was measured by spectrophotometer at 750 nm. Amount of protein in different fractions were estimated from the standard curve (Figure 24). Result is shown in Table 20. In each case, 20 µl of sample (fractions) was taken with 980 µl of distilled water (total volume 1ml) for estimation of soluble protein. Fraction 3 (F_3) contains the highest water soluble protein (4932.14 µg/ml) and fraction 1 contains the lowest water soluble protein (1182.14µg/ml).

Table 20. Soluble proteins of different fractions of *Azadirachta indica* A. juss.leaf extract

Sl No.	Name	Optical density at 750 nm	Total amount of protein (µg/ml) per fraction
1	Fraction-1	0.096	1182.14
2	Fraction-2	0.115	1860.71
3	Fraction-3	0.201	4932.14
4	Fraction-4	0.133	2503.57
5	Fraction-5	0.129	2360.71

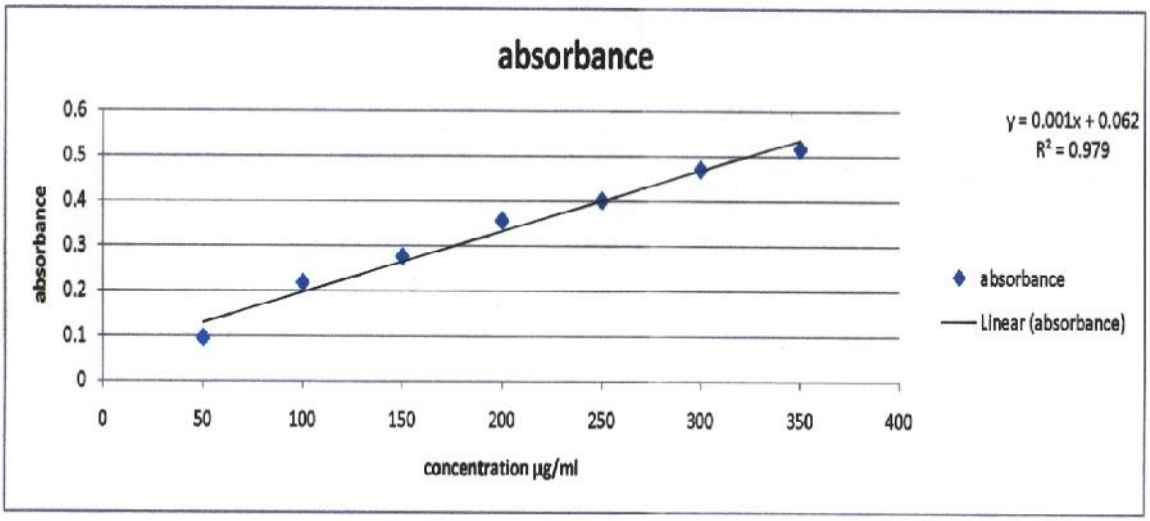


Figure 24. Amount of soluble proteins µg/ ml from different fractions of *Azadirachta indica* A. Juss.

4.8.2. Protein analysis for *Azadirachta indica* A. juss.using Polyacrylamide Gel Electrophoresis

To determine the molecular weight SDS PAGE analysis is very reliable. Since the mobility of a substance in a gel is influenced by both charge and size, in SDS PAGE samples are treated in order to have a uniform charge, so electrophoretic mobility in SDS PAGE depends mainly on size. SDS PAGE is carried out in the presence of an anionic detergent sodium dodecyl sulfate (SDS) and a reducing agent mercaptoethanol (BME). SDS disrupts the secondary, tertiary and quaternary structure of the protein to produce a linear polypeptide chain coated with negatively charged SDS molecules. BME contributes with protein denaturation by reducing all disulfide bonds. On the other hand in Native PAGE proteins are prepared in a non-reducing and non-denaturing sample buffer (neither SDS nor BME in the sample buffer and in the gel), which maintains both the proteins' secondary structure and native charge density, so this technique can could be used, for example, to know the aggregation state of a protein .

For proteomic analysis of *Azadirachta indica* A. juss, total protein was run in native condition. Presences of protein from the different fractions of *Azadirachta indica* A. Juss.leaf extract were examined using native polyacrylamide gel electrophoresis. The presence protein of in terms of different intensities of the bands were termed as very dark, dark, light and very light. Similar protein banding pattern of SDS-PAGE was also observed in case of native gel. The different sizes of proteins as detected on native gel shown Figure 25 where two protein bands are present in all of five different fractions (i. e. 70 kDa and 37 kDa). From the figure it can be inferred that fraction 3 might contain active proteins because this fraction has more protein bands (i. e.70 kDa, 40 kDa and 37 kDa) with prominent intensity.

From the native PAGE the proteins were appeared in their folded structures. Therefore, to observed the unfold structure/linear froms of protein SDS PAGE was carried out and different sizes of proteins were detected on polyacrylamide gel (Figure 26). Nine frequent bands were determined from all the five different fractions. Based on the figure it can be inferred that fraction 3 might contain active proteins because this fraction hasmore prominent protein bands.

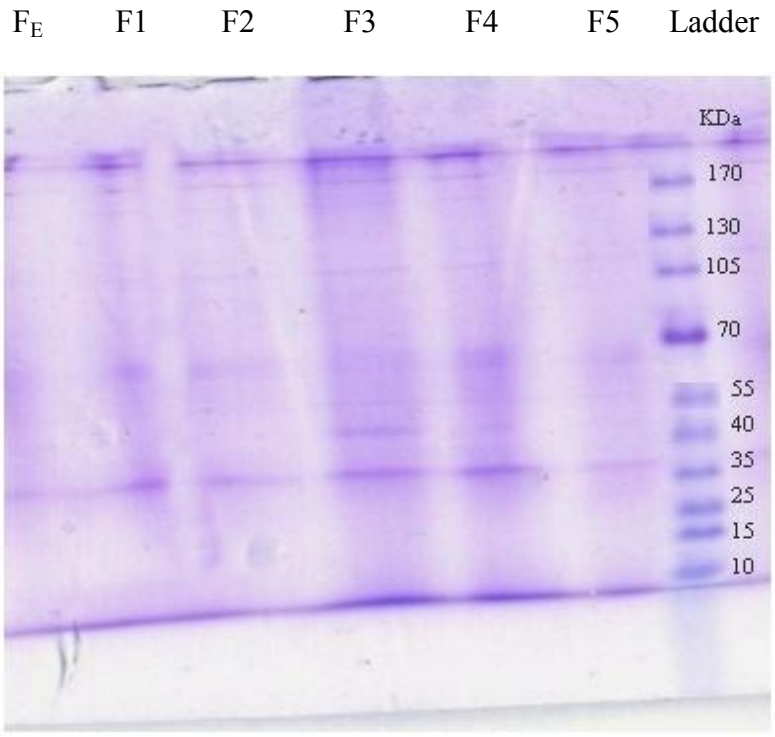


Figure 25. Protein analysis of the different fractions of *Azadirachta indica* A. juss.proteins on native polyacrylamide gel. Lanes F1, F2, F3, F4, F5 are indicating five different fractions.

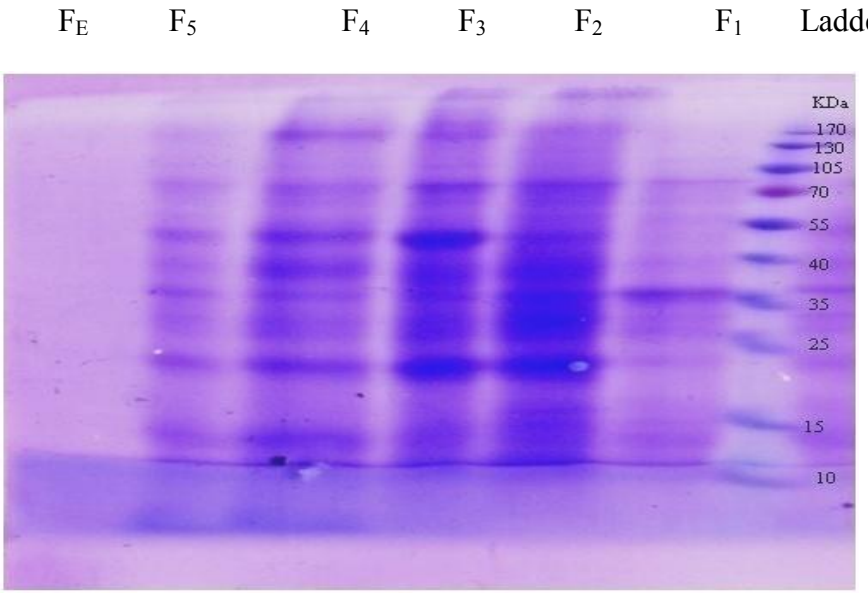


Figure 26.Total protein of the different fractions of *Azadirachta indica* A. juss.proteins on polyacrylamide gel (SDS PAGE). Lane 1, 2, 3, 4, 5, 6 corresponding to five fractions as F_1, F_2, F_3, F_4, F_5 and $F_{E,}$ respectively.

4.8.3. Effects of different fractions of *Azadirachta indica* A. juss. on *Neurospora crassa*

The possible reason behind the antifungal activity of *Azadirachta indica* may be proteins or non-proteins fraction or both. To find out which fraction is specifically been the active proteins, effects of different fractions has been demonstrated. Previously, in our study it was observed that antifungal activity was positvely dependent on the concentration of protein. Antifungal activity was tested with 50 µg to 200 µg of protein. Intrestingly, it was observed that proteins of different fractions were able to inhibit the radial gowth of *Neurospora crassa* but 200 µg proteins of each fraction showed the antifungal activity. 200 µg protein were mixed in separate petridish and since non-protein part does not contain any protein, 200 µl elute was used in this case. The petridishes were marked as F_0 (no protein only VM), F_1 (169.18µl elute contain 200 µg protein), F_2 (107.48 µl elute contain 200 µg protein), F_3 (40.55 µl elute contain 200 µg protein), F_4 (79.88 µl elute contain 200 µg protein, F_5 (84.72 µl elute contain 200 µg protein) F_E (200 µl end product). 10 ml molten VM was added in each petridish. When media solidified, the spore of *Neurospora crassa* was inoculated at the centre of the media. Plates were incubated at 25°C and radial growth was measured after 24 hours. Radial growth was observed at least three times and average length of radial growth (cm) has been summarized in table 21. According to the table it was observed that fraction 3 (F_3) and fraction 4 (F_4) had no growth and end product (the remaining soup after 100% saturation) had no activity which corresponding to F_0 where no protein was added. But the growth was little bit showen than the F_0 plate (figure 27).

Table 21. Radial growth of Em 'a' on VM containing different fractions of *Azadirachta indica* A. Juss. leaves

Plate No.and conc. of the solutions	Observation after 24 hours of mean radial growth (cm)
F_o(no protein only VM)	2.7
F_1(200 µg protein)	0.6
F_2(200 µg protein)	0.3
F_3(200 µg protein)	No growth
F_4(200 µg protein)	No growth
F_5(200 µg protein)	0.1
F_E (200 µl end product)	2.7

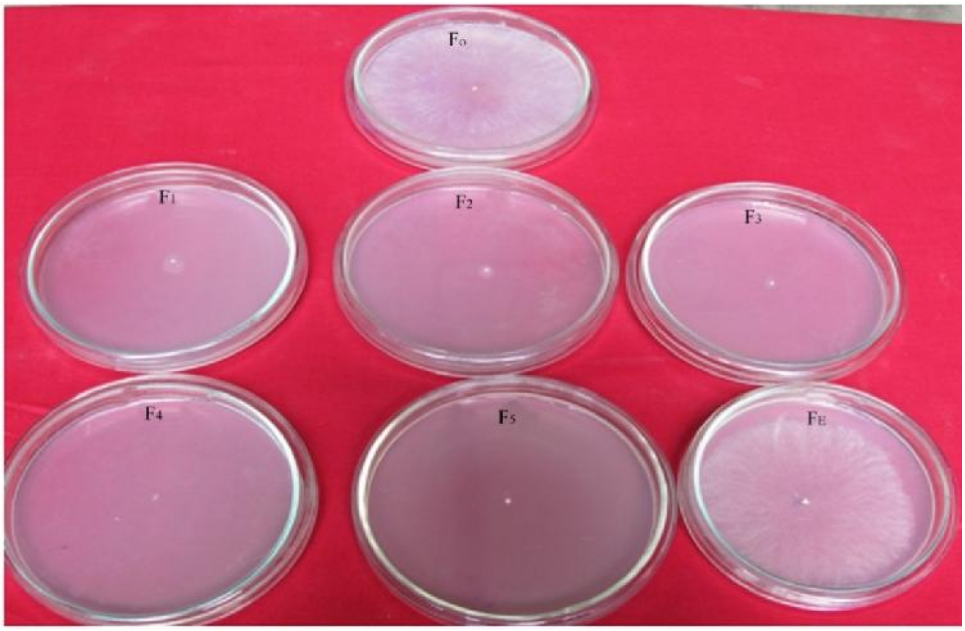


Figure 27. Comparative radial growth of Em 'a' on VM containing different fractions of proteins *Azadirachta indica* A. Juss. leaf. Where Fo= control plate (only VM), F_1=fraction 1, F_2= fraction 2, F_3= fraction 3, F_4= fraction 4, F_5= fraction 5 and F_E= end prouct.

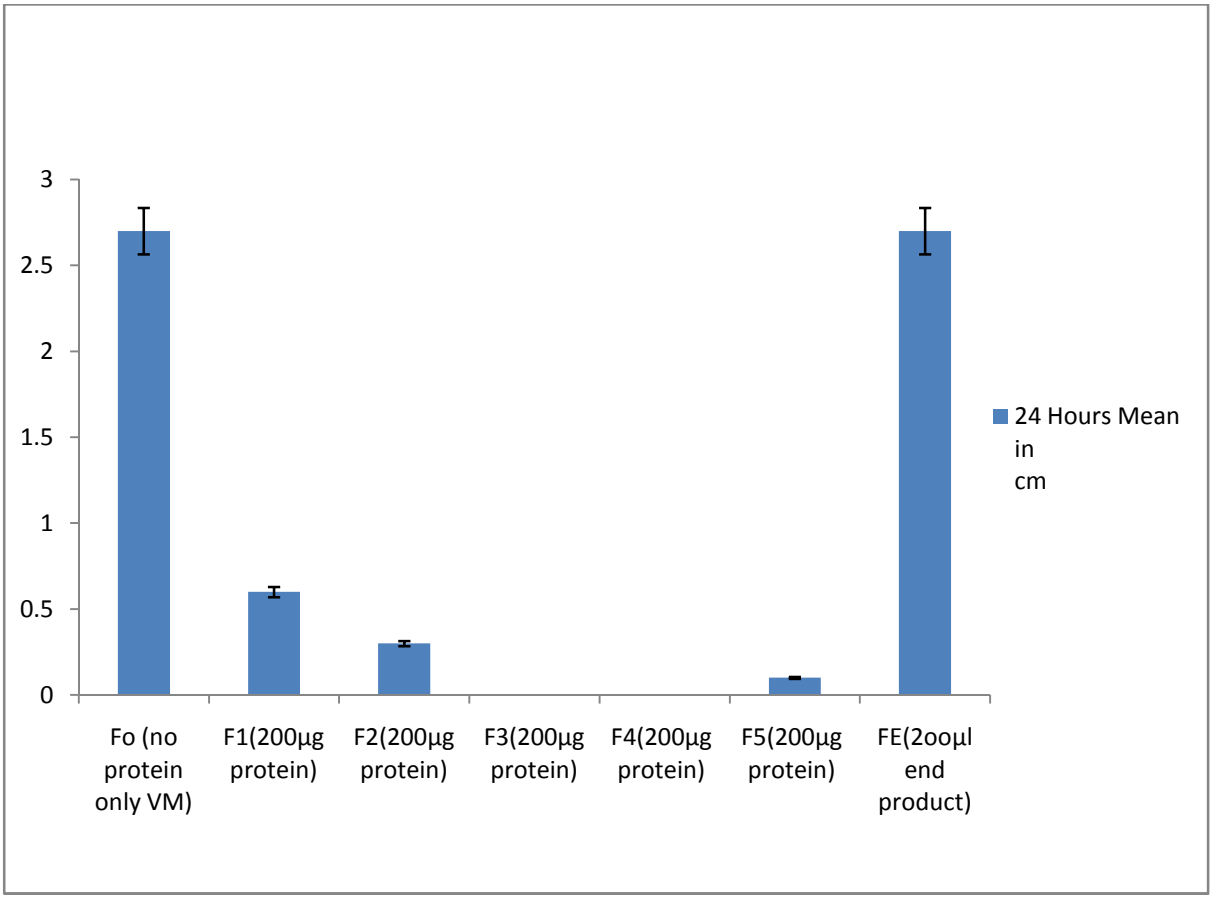


Figure 28. Graph showing radial growth of *Neurospora crassa* with protein of different fractions of *Azadirachta indica* A. Juss. leaf (after 24 hours).

4.8.4. Effects of different fractions of *Azadirachta indica* A. juss. on *Helminthosporium oryzae*

The possible reason behind the antifungal activity of *Azadirachta indica* may be proteins or non-proteins fraction or both. To find out which fraction is specifically been the active proteins, effects of different fractions has been demonstrated. Previously, in our study it was observed that antifungal activity was positvely dependent on the concentration of protein. Antifungal activity was tested with 50 µg to 200 µg of protein. Intrestingly, it was observed that proteins of different fractions were able to inhibit the radial gowth of *Helminthosporium oryzae* but 200 µg proteins of each fraction showed the antifungal activity. 200 µg protein were mixed in separate petridish and since non-protein part does not contain any protein, 200 µl elute was used in this case. The petridishes were marked as F_0 (no protein only PDA), F_1 (169.18 µl elute contain 200 µg protein), F_2 (107.48 µl elute contain 200 µg protein), F_3 (40.55 µl elute contain 200 µg protein), F_4 (79.88 µl elute contain 200 µg protein), F_5 (84.72 µl elute contain 200 µg protein) and F_E (200 µl end product). 10 ml molten VM was added in each petridish. When media became solid the spore of *Helminthosporium oryzae* was inoculated at the centre of the media. Plates were incubated at 25°C and measured the radial growth after 24 hours. Radial growth was observed at least three times and average length of radial growth in centimeter has been summarized in table 22. According to the table it was observed that fraction 3 (F_3) and fraction 4 (F_4) had no growth and end product (after 100% saturation the remaining soup) had no activity corresponding with F_0 where no protein was added. But the growth was little bit showen than the F_0 plate (Figure 29).

Table 22. Radial growth of *Helminthosporium oryzae* on PDA containing different fractions of *Azadirachta indica* A. Juss. leaf

Plate No and concentration of the solutions	Observation after 24 hours growth (cm)
F_o (no protein only PDA)	2.0
F_1(200 µg protein)	0.6
F_2(200 µg protein)	0.3
F_3(200 µg protein)	No growth
F_4 (200 µg protein)	No growth
F_5 (200 µg protein)	0.1
F_E (200µl end product)	2.0

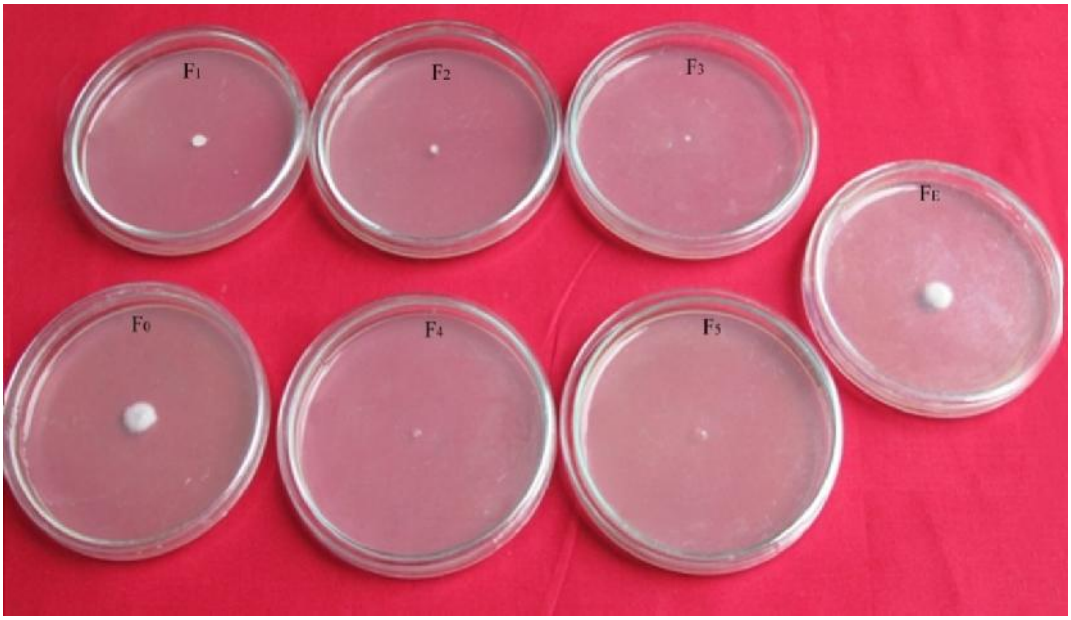


Figure 29. Comparative radial growth *of Helminthosporium oryzae* on PDA containing proteins of different fractions of *Azadirachta indica* A. Juss. leaf Fo = control plate (only PDA), F_1=fraction 1, F_2= fraction 2, F_3= fraction 3, F_4= fraction 4, F_5= fraction 5, F_E= end product.

.

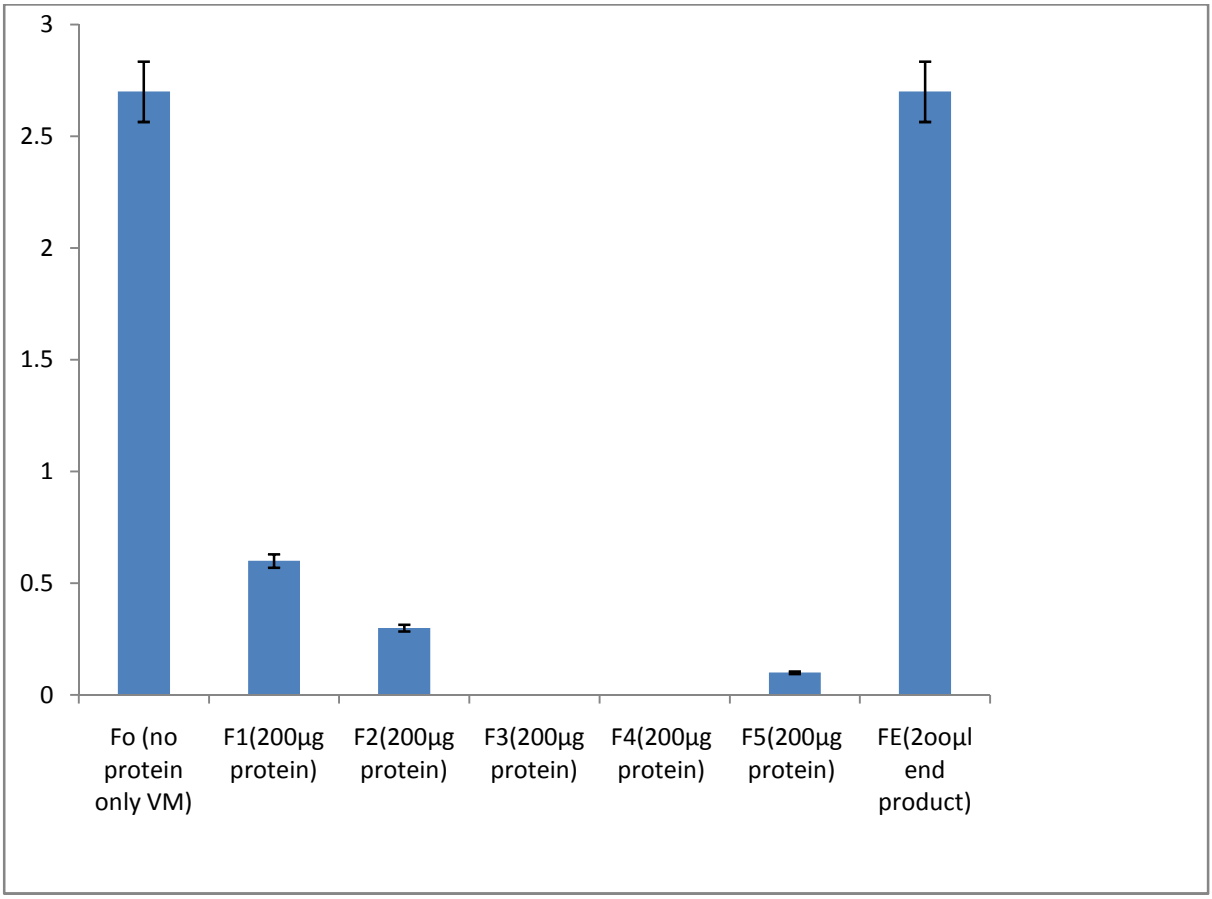


Figure 30. Graph showing radial growth of *Helminthosporium oryzae* with proteins of different fractions of *Azadirachta indica* A. Juss. leaf (after 24 hours)

4.8.5. Effects of specific protein of *Azadirachta indica* A. Juss. on *Neurospora crassa* and *Helminthosporium oryzae*

It was observed from the native PAGE, fraction 3 has protein bands at size 40 kDa and 37 kDa (Figure 25). These proteins may be the probable candidates, which might have specific antifungal activity on *Neurospora crassa* and *Helminthosporium oryzae*. To find out these two specific protein bands were cut and directly used to growth media.The activity of polyacrylamide was used as negative control. Figure 37 and 38 showed that both proteins have antifungal activity against *Neurospora crassa* and *Helminthosporium oryzae,* respectively. On the other hand, polyacrylamide gel had no antifungal activity on the growth of *Neurospora crassa* and *Helminthosporium oryzae* (Figure 31 and 32).

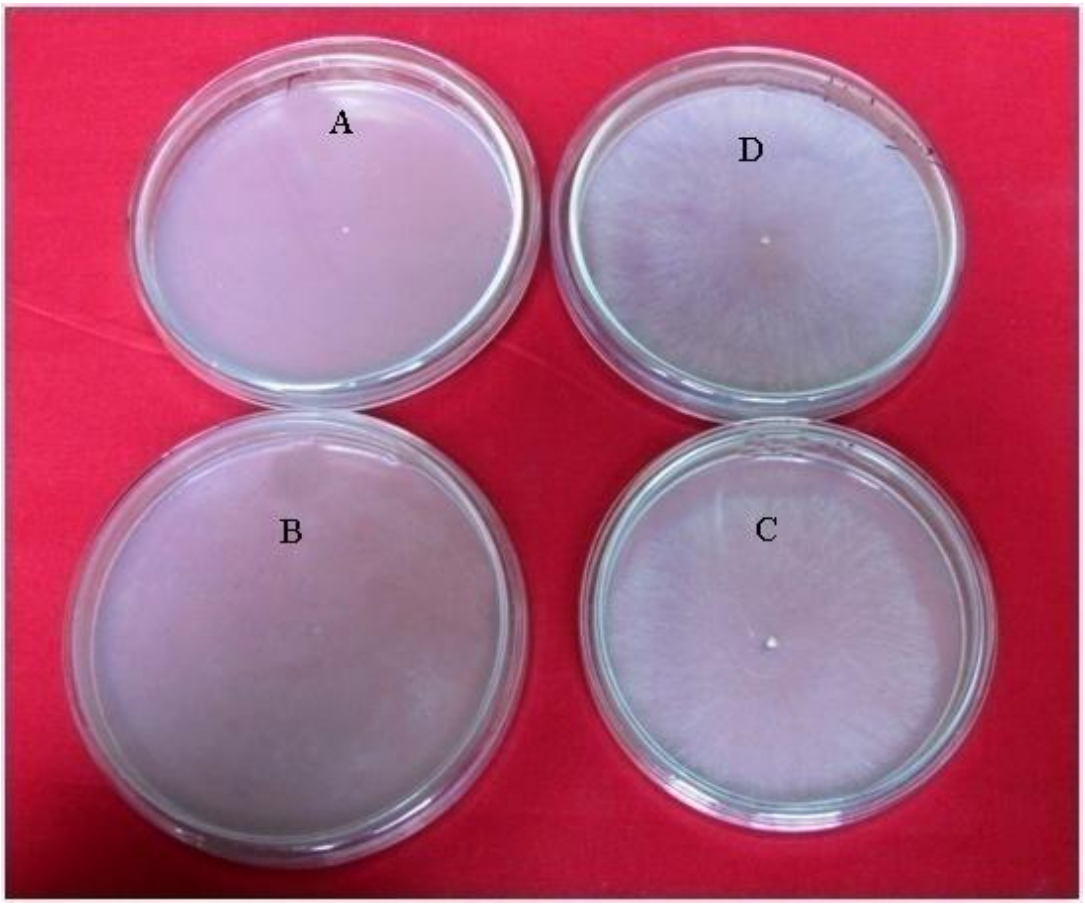


Figure 31. Antifungal activity of the specific proteins present in the fraction no. 3.A= 40 kDa protein band and B= 37 kDa protein band C= polyacrlamide gel D= control plate (only VM).

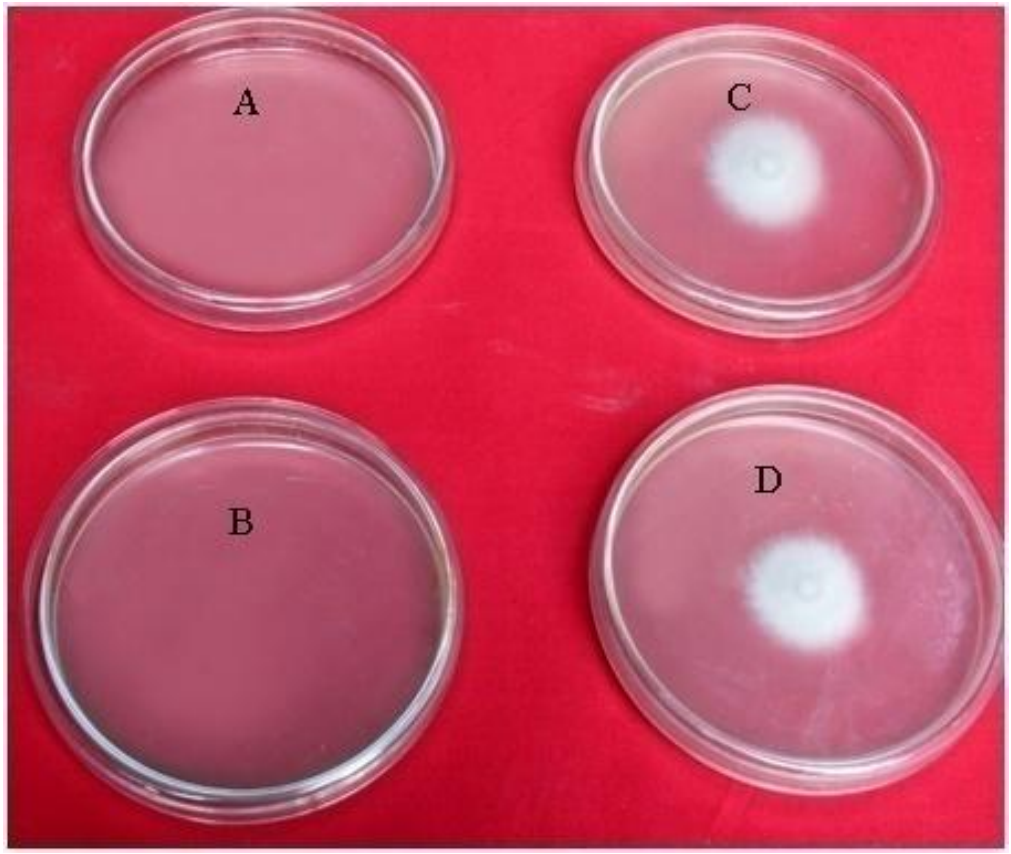


Figure 32. Antifungal activity of the specific proteins present in the fraction no. 3. A= 37 kDa protein band and B= 40 kDa protein band C= polyacrlamide gel D= control plate (only PDA).

DISCUSSION

The application of chemical fungicides to control the plant diseases has many drawbacks and is hazardous to the environment. Hence, to find out an alternative, locally available plants that possess medicinal properties were investigated. Many workers have reported antifungal properties of many plants against many fungal diseases. Bisht and Khulbe (1995) reported that mycelial growth of *D. oryzae* inhibited by *Juglans regia* (64.11%) in an *in vitro* experiment. Many workers have already reported the antifungal and antimicrobial effect of different medicinal plants (Dabur *et al.* 2004, Sivanathan M 2013, Sanguri *et al*. 2012).

Previous studies provide evidence that some medicinal plants might be the potential sources of new antifungal agents, even antibiotic-resistant strains (Jagtap *et al.* 2009). From the present study, it is concluded that locally available plant like *Ocimum sanctum* Linn., *Centella asiatica* (Linn.) Urban*., Coccinia cordifolia* (Linn.) Cogn*., Andrographis paniculata* (Burm.f.) Wall. may be use to control the *Neurospora crassa.* Hence, locally available plants need to be identified as an alternative of chemical fungicides for safe and effective control of plant diseases.

Using leaf extracts of *Ocimum sanctum* Linn., *Centella asiatica* (Linn.) Urban*., Coccinia cordifolia* (Linn.) Cogn*., Andrographis paniculata* (Burm.f.) Wall. at 25%, 50%,75% and 100% concentration, it was found that antifungal activity of these plant extracts increased with increasing concentration. (Table 8 to 15). It was found that although not promising but still the antifungal effect of the plant extract persisted even at 25% concentration. It was evident from table 16, 17 and 18, 19 that the radial growth of *Neurospora crassa* was reduced as compared to control. 100% concentration was found to be more effective than other concentration. This observation suggested that the plant extracts were found to be promising against the test pathogens and could be increased further by using these plant extracts at higher concentrations.

A large number of chemicals have been developed for the control of plant diseases. But due to overgrowing awareness of the hazardous side effects of these chemicals, tremendous emphasis is given to the use of biocontrol agents. Now major challenge in the field of plant pathology to introduce some ecofriendly and safe alternative control strategies for agriculture, which led researchers to turn their attention to plants and microorganisms as sourceses of biocontrol agents. As the source of biocontrol agent, *Andrographis paniculata* (Burm.f.) Wall. *Azadirachta indica* A. Juss has already emerged at the top of the list with the highest

potential. It contains at least 35 biologically active ingradients of which nimbin and azadirachtin (Pennigton TD *et al.* 1981) are the most active fungicidal ingradients and are present predominantly in the seeds, leaves and other parts of the neem tree (Mulla *et al.* 1999). In this study the antifungal effect of *Andrographis paniculata* (Burm.f.) Wall. *Azadirachta indica* A. Juss was studied in an *in vitro* culture medium of *Helminthosporium oryzae* and *Neurospora crassa* and subsequent molecular characterization of *Andrographis paniculata* (Burm.f.) Wall. *Azadirachta indica* A. Juss leaf extracts wasconducted for the analysis of its antifungal activity.

During this study, leaf extracts of *Ocimum sanctum* Linn. (Tulshi), *Coccinia cordifolia (*Linn). Cogn (Telakucha), *Andrographis paniculata* (Burm.f.) Wall. (Kalomegh), *Centella asiatica* (Linn.) Urban. (Thankuni) and *Azadirachta indica* A. Juss. (Neem) were tested for antifungal activity at different concentrations. Among all the tested plants, different concentrations of leaf extract of *Azadirchta indica* A. Juss. showed best antifungal activity on radial growth of *Neurospora crassa* (Table 16and 17) and *Helminthosporium oryzae* (Table 18 and 19). From the data, it is observed that by increasing the concentrations the radial growth of *Helminthosporium oryzae* were reduced in comparison to control. This observation suggested that antifungal activities of the plant extracts against test pathogens could be increased further at higher concentrations.

Plant contains various primary and secondary metabolites. Protein is secondary metabolite, whichacts as inhibitor of the fungal growth. According to the circumstances, *Andrographis paniculata* (Burm.f.) Wall. and *Azadirachta indica* A. Juss protein were extracted in five different fractions. The proteins analyzed by Poly Acrylamide Gel Electrophoresis (native PAGE). Poly Acrylamide Gel Electrophoresis separates molecules in complex mixtures according size and charge. During electrophoresis there is an intricate interaction of samples, gel matrix buffer and electric current resulting in separate bands of individual protein.

Soluble protein contents different fractions of *Azadirachta indica* A. Juss. were studied. Soluble proteins were estimated according to Lowry *et al*. (1951), using Bovine Serum Albumin (BSA) as standard. Electrophoresis of proteins techniques have been prove to be powerful tool for a wide range of biological investigations. Polyacrylamide gel electrophoresis (PAGE) can be used for many kinds of phytochemistry investigations.

Proteins can serve as unique markers and can be used aschemotaxonomy and in studying differentiation protein analysis (Hames BD 1990, Marrs SB 1993).

The results from the present investigation revealed distinct banding patterens among the supplied protein samples. The different protein activity was determind in terms of band intensity and termed as very dark, dark, light and very light. It indicated that the percentage (%) of protein content in the different fractions of *Azadirachta indica* A. Juss. leaves. The darker region indicates high percentage (%) of protein content than the lighter one. The activity of these proteins as detected on polyacrylamide gels is shown in figure 25 and 26.

Presence of protein from five different fractions was examined using polyacrylamide gel electrophoresis (SDS PAGE). The different protein activity was determind in terms of band intensity and were termed as very dark, dark, light and very light. It indicated that the percentage (%) of protein content in the different fractions of *Azadirachta indica* A. Juss. leaves. The darker region indicates high percentage (%) of protein content than the lighter one. The activity of these proteins as detected on polyacrylamide gels is shown in figure 23 (a, b, c, d, e). From polyacrylamide gel electrophoresis it was revealed that fractions 3 (protein size 40 kDa and 37 kDa bands) contain active proteins. These two proteins were capable of inhibiting the tested fungal growth. Previous studies revealed similar kind of result with alkaloids (Conventry E and Allan J 2001).

The present study is an attempt to screen selected medicinal plants. This experiment clearly revealed the presence of antifungal properties in the test plants. This may be due to the potential compounds were present in the extract of the *Azadirachta indica* A Juss. leaves. These plants could be potential sources of new phytotoxic and antifungal agents. As effective fungicidal activities were observed, more research should be directed towards the isolation of fungicidal bioactive compounds as well as further field trails could be carried out to confirm the present finding.

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APPENDIX

ABBREVIATION

C = Centrigrade/ Celsius

Cm = Centimeter(s)

et al. = et alil and others

etc = et cetra, and the rest

Fig/s = Figure/ figures

g/ gm = gram(s)

M = Molar

Mg/l = Miligram per liter

Mg = Miligram

mM = Millimolar

No = Number

OD = Optical density

pH = Negative logarithm of hydrogen

% = Percentage

µ = Micron

µl = Micro liter

µg = Micro gram

V = Volt

AP = Ammonium per sulphate

W/V = Weight per volume

Em = Emerson stock

Ema = Emerson stock mating type 'a'

EmA = Emerson mating type 'A'

VM = Vogel's minimal medium

F_1 = Fraction one

F_2 = Fraction two

F_3 = Fraction three

F_4 = Fraction four

F_5 = Fraction five

F_E = End product

F_0 = No protein only VM medium

F_0 = No protein only PDA medium

Nm = Nano meter

Ml =Milliliter

PDA = Potato Dextrose Agar

O1 = 25% crude extract of *Ocimum sanctum*

O2 = 50% crude extract of *Ocimum sanctum*

O3 = 75% crude extract of *Ocimum sanctum*

O4 = 100% crude extract of *Ocimum sanctum*

C1 = 25% crude extract of *Coccinia cordifolia*

C2 = 50% crude extract of *Coccinia cordifolia*

C3 = 75% crude extract of *Coccinia cordifolia*

C4 = 100% crude extract of *Coccinia cordifolia*

T1 = 25% crude extract of *Centella asiatica*

T2 = 50% crude extract of *Centella asiatica*

T3 = 75% crude extract of *Centella asiatica*

T4 = 100% crude extract of *Centella asiatica*

A1 = 25% crude extract of *Andrographis paniculata*

A2 = 50% crude extract of *Andrographis paniculata*

A3 = 75% crude extract of *Andrographis paniculata*

A4 = 100 %crude extract of *Andrographis paniculata*

N1 = 25% crude extract of *Azadirachta indica*

N2 = 50% crude extract of *Azadirachta indica*

N3 = 75% crude extract of *Azadirachta indica*

N4 = 100% crude extract of *Azadirachta indica*

TEMED = Tetramethylethylenediamine

SDS = Sodium Dodecyl Sulphate

HCl = Hydrocloric Acid

KCl = Potassium chloride

NACl = Sodium Chloride

Ib = Pound

kDa = Kilodalton

PAGE = Polyacrylamide gel electrophoresis

PBS =Phosphate Bufferer Saline

rpm = Rotation per minute

BSA = Bovine serum albumin